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<p>(54) Title: POLYKETIDE-ASSOCIATED SUGAR BIOSYNTHESIS GENES (57) Abstract  The present invention provides isolated polynucleotides from <i>Saccharomyces erythraea</i> that encode enzymes involved in the biosynthesis of polyketide-associated sugars. Methods of using the polynucleotides to produce novel glycosylation modified polyketides are also provided.</p>		

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## POLYKETIDE-ASSOCIATED SUGAR BIOSYNTHESIS GENES

This application claims the benefit of U.S. Serial No. 08/576,626 filed December 21, 1995, now pending.

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### Field of the Invention

The present invention relates to methods for directing the biosynthesis of specific polyketide analogs by genetic manipulation. In particular, sugar biosynthesis genes are manipulated to produce precise, novel glycosylation-modified macrolides of predicted structure.

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### Background of the Invention

Polyketides are a large class of natural products that includes many important antibiotic, antifungal, anticancer, and anti-helminthic compounds such as erythromycins, amphotericins, daunorubicins, and avermectins. Their synthesis proceeds by an ordered condensation of acyl esters to generate carbon chains of varying length, side chain, and reduction pattern that are differentially cyclized and subsequently modified to give the mature polyketides. For many polyketides, maturation includes the addition of one or more sugar residues to the cyclized carbon chain. The sugar residues are frequently critical to the biological activity of the mature polyketide.

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*Streptomyces* and the closely related *Saccharopolyspora* genera are prodigious producers of polyketide metabolites. Because of the commercial significance of these compounds, a great amount of effort has been expended in the study of *Streptomyces* genetics. Consequently, much is known about *Streptomyces* and several cloning vectors exist for introducing DNA into these organisms.

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Although many polyketides have been identified, there remains the need to obtain novel glycosylation modified (as defined herein) polyketide structures with enhanced properties. Current methods of obtaining such molecules include screening of biological samples and chemical modification of existing polyketides, both of which are costly and time consuming. Current screening methods are based on gross properties of the molecule, i.e. antibacterial, antifungal activity, etc., and both *a priori* knowledge of the structure of the molecules obtained or predetermination of enhanced properties are virtually impossible. Standard chemical modification of existing structures has been successfully employed, but is limited by the number of types of compounds obtainable. Furthermore, the poor yield of multistep chemical syntheses often limits the practicality of this approach. The following modifications to sugar residues bound to polyketides are particularly difficult or inefficient at the present time: change the stereochemistry of specific hydroxyl or methyl groups, change the oxidation state of specific hydroxyl groups, and deoxygenation of specific carbons. Accordingly, there exists a need to obtain molecules wherein such changes are specified and

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performed which would represent an improvement in the technology to produce altered glycosylation-modified polyketide molecules with predicted structure.

The present invention overcomes these problems by providing the genetic sequence of sugar biosynthesis genes involved in the biosynthesis of polyketide-associated sugars.

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#### Summary of the Invention

In one aspect, the present invention provides an isolated single or double stranded polynucleotide, typically DNA, having a nucleotide sequence which comprises (a) a nucleotide sequence selected from the group consisting of (i) the sense sequence of FIG. 4A (SEQ ID NO:1) from about nucleotide position 54 to about nucleotide position 1136; (ii) the sense sequence of SEQ ID NO:1 from about nucleotide position 1147 to about nucleotide position 2412; (iii) the sense sequence of SEQ ID NO:1 from about nucleotide position 2409 to about nucleotide position 3410; (iv) the sense sequence of FIG. 4B (SEQ ID NO:2) from about nucleotide position 80 to about nucleotide position 1048; (v) the sense sequence of SEQ ID NO:2 from about nucleotide position 1048 to about nucleotide position 2295; (vi) the sense sequence of SEQ ID NO:2 from about nucleotide position 2348 to about nucleotide position 3061; (vii) the sense sequence of SEQ ID NO:2 from about nucleotide position 3214 to about nucleotide position 4677; (viii) the sense sequence of SEQ ID NO:2 from about nucleotide position 4674 to about nucleotide position 5879; (ix) the sense sequence of SEQ ID NO:2 from about nucleotide position 5917 to about nucleotide position 7386; and (x) the sense sequence of SEQ ID NO:2 from about nucleotide position 7415 to about nucleotide position 7996; (b) sequences complementary to the sequences of (a); (c) sequences that, on expression, encode a polypeptide encoded by the sequences of (a); and (d) analogous sequences that hybridize under stringent conditions to the sequences of (a) and (b). A preferred molecule is a DNA molecule. In another embodiment, the polynucleotide is an RNA molecule.

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In another embodiment, a DNA molecule of the present invention is contained in an expression vector. The expression vector preferably further comprises an enhancer-promoter operatively linked to the polynucleotide. In a preferred embodiment, the DNA molecule in the vector is one of the preferred sequences mentioned above. In an especially preferred embodiment, the DNA molecule in the vector is the sequence of SEQ ID NO:2 from about nucleotide position 80 to about nucleotide position 1048.

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The present invention still further provides for a host cell transformed with a polynucleotide or expression vector of this invention. Preferably, the host cell is a bacterial cell selected from the group consisting of *Saccharopolyspora spp.*, *Streptomyces spp.* and *E. coli*.

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The present invention also provides methods to produce novel glycosylation modified

polyketide structures by designing and introducing specified changes in the DNA governing the synthesis and attachment of sugar residues to polyketides. According to one method, the biosynthesis of specific glycosylation-modified polyketides is accomplished by genetic manipulation of a polyketide-producing microorganism comprising the steps of isolating a  
5 sugar biosynthesis gene-containing DNA sequence from those described above; identifying within the gene-containing DNA sequence one or more DNA fragments responsible for the biosynthesis of a polyketide-associated sugar or its attachment to the polyketide; creating one or more specified changes into the DNA fragment or fragments, thereby resulting in an altered DNA sequence; introducing the altered DNA sequence into a polyketide-producing  
10 microorganism to replace the original sequence whereby the altered DNA sequence, when translated, results in altered enzymatic activity capable of effecting the production of the specific glycosylation-modified polyketide; growing a culture of the altered polyketide-producing microorganism under conditions suitable for the formation of the specific glycosylation-modified polyketide; and isolating said specific glycosylation-modified  
15 polyketide from the culture.

In a second method the biosynthesis of specific glycosylation-modified polyketides is accomplished by isolating a sugar biosynthesis gene-containing DNA sequence from from those described above; identifying within the gene-containing DNA sequence one or more  
20 DNA fragments responsible for the biosynthesis of a polyketide-associated sugar or its attachment to the polyketide; reversing the strand orientation of the DNA fragment or fragments, thereby resulting in an altered DNA sequence which, when transcribed, results in production of an antisense mRNA; introducing the altered DNA sequence into a polyketide-producing microorganism having an mRNA capable of binding to the antisense mRNA which results in altered enzymatic activity capable of effecting the production of the specific  
25 glycosylation-modified polyketide; growing a culture of the altered polyketide-producing microorganism under conditions suitable for the formation of the specific glycosylation-modified polyketide; and isolating the specific glycosylation-modified polyketide from the culture.

In a third method the biosynthesis of specific glycosylation-modified polyketides is  
30 accomplished by isolating a sugar biosynthesis gene-containing DNA sequence from from those described above; identifying within the gene-containing DNA sequence one or more DNA fragments responsible for the biosynthesis of a polyketide-associated sugar or its attachment to the polyketide; introducing the DNA fragment or fragments into a polyketide-producing microorganism whereupon transcription and translation of the DNA fragment or  
35 fragments generate an altered polyketide-producing microorganism that is capable of producing the specific glycosylation-modified polyketide; growing a culture of the polyketide-producing microorganism containing the DNA fragment or fragments under

conditions suitable for the formation of the specific glycosylation-modified polyketide; and isolating the specific glycosylation-modified polyketide from the culture.

Preferably, the sugar biosynthesis gene-containing DNA sequence of the processes described above comprises genes which encode an enzymatic activity involved in the biosynthesis of L-mycarose and/or D-desosamine. More preferably, the sugar biosynthesis gene-containing DNA sequence comprises the sequence of SEQ ID NO:2 from about nucleotide position 80 to about nucleotide position 1048.

The present invention is especially useful in manipulating sugar biosynthesis genes from *Streptomyces* and *Saccharopolyspora*, organisms that provide over one-half of the clinically useful antibiotics.

#### Brief Description of the Drawings

FIG. 1A illustrates the organization of the erythromycin biosynthetic gene cluster and the genetic designations of the biosynthetic genes; FIG. 1B illustrates an abbreviated erythromycin biosynthetic scheme that broadly associates the biosynthetic genes with their role in erythromycin biosynthesis. Seven *eryB* genes, *eryBI* - *eryBVII*, are responsible for the biosynthesis of L-mycarose or its attachment to the erythronolide B ring, and six *eryC* genes, *eryCI* - *eryCVI*, are responsible for the biosynthesis of D-desosamine or its attachment to 3- $\alpha$ -mycarosylerythronolide B. The dashed arrows indicate that the pathway through erythromycin B is not the principal natural biosynthetic route to erythromycin A.

FIG. 2 illustrates the proposed scheme for the biosynthesis of L-mycarose and the *eryB* genes responsible for the specific steps.

FIG. 3 illustrates the proposed scheme for the biosynthesis of D-desosamine and the *eryC* genes responsible for the specific steps.

FIG. 4A(1-4) illustrates the nucleotide sequence (SEQ ID NO:1) of the sugar biosynthesis genes *eryCII* (coordinates 54-1136), *eryCIII* (coordinates 1147-2412), and *eryBII* (coordinates 2409-3410), with corresponding translation of the open reading frames (SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5 respectively). Standard one letter codes for the amino acids appear beneath their respective nucleic acid codons as described herein.

FIG. 4B(1-9) illustrates the nucleotide sequence (SEQ ID NO:2) of the sugar biosynthesis genes *eryBIV* (coordinates 80-1048), *eryBV* (coordinates 1048-2295), *eryCVI* (coordinates 2348-3061), *eryBVI* (coordinates 3214-4677), *eryCIV* (coordinates 4674-5879), *eryCV* (coordinates 5917-7386), and *eryBVII* (coordinates 7415-7996) with corresponding

translation of the putative open reading frames (SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11 and SEQ ID NO:12 respectively). Standard one letter codes for the amino acids appear beneath their respective nucleic acid codons as described herein.

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FIG. 5A illustrates the amino acid sequence identity between the sugar biosynthesis enzyme encoded by the *eryBIV* gene of *Sac. erythraea* (SEQ ID NO:6) and the sugar biosynthesis enzymes encoded by the *ascF* gene of *Yersinia pseudotuberculosis* [Thorson *et al.*, *J. Bacteriol.*, 176:5483 (1994)], (SEQ ID NO:13), the *rfbJ* gene of *Salmonella enterica* [Jiang *et al.*, *Mol. Microbiol.*, 5:695 (1991)]. (SEQ ID NO:14), the *strL* gene of *Streptomyces griseus* [Pissowatzki *et al.*, *Mol. Gen. Genet.* 241:193 (1993)] (SEQ ID NO:15) and the *galE* gene of *Escherichia coli* [Lemaire and Hill, *Nucl. Acids Res.* 14:7705 (1986)] (SEQ ID NO:16). In this and all other Figures in which amino acid sequence identity is compared capitalized letters represent consensus (identical) amino acids between species or amino acids which are conservative substitutions for the consensus residues. Also in each Figure, the sequence identified as "consensus" is merely a convenient representation of conserved amino acids and is not intended as a representation of any existing polypeptide sequence.

FIG. 5B illustrates the amino acid sequence identity between the sugar biosynthesis enzyme encoded by the *eryBVII* gene of *Sac. erythraea* (SEQ ID NO:12) and the sugar biosynthesis enzymes encoded by the *strM* gene of *Streptomyces griseus* [Pissowatzki *et al.*, *Mol. Gen. Genet.* 241:193 (1993)] (SEQ ID NO:17), the *rfbC* gene of *Salmonella enterica* [Jiang *et al.*, *Mol. Microbiol.*, 5:695 (1991)] (SEQ ID NO:18), the *rfbF* gene of *Yersinia enterocolitica* [Zhang *et al.*, *Mol. Microbiol.*, 9:309 (1993)] (SEQ ID NO:19), and the *ascE* gene of *Yersinia pseudotuberculosis* [Thorson *et al.*, *J. Bacteriol.*, 176:5483 (1994)] (SEQ ID NO:20).

FIG. 5C illustrates the amino acid sequence identity between the sugar biosynthesis enzyme encoded by the *eryCIV* gene of *Sac. erythraea* (SEQ ID NO:10) and the sugar biosynthesis enzymes encoded by the *eryCI* gene of *Sac. erythraea* [Dhillon *et al.*, *Mol. Microbiol.*, 3:1405 (1989)] (SEQ ID NO:21), the *ascC* gene of *Yersinia pseudotuberculosis* [Weigel *et al.*, *Biochemistry*, 31:2129 (1992), Thorson *et al.*, *J. Am. Chem. Soc.*, 115:6993 (1993), Thorson *et al.*, *J. Bacteriol.*, 176:5483 (1994)] (SEQ ID NO:22), the *dnrJ* gene of *Streptomyces peucetius* [Stutzman-Engwall *et al.*, *J. Bacteriol.*, 174:144 (1992)] (SEQ ID NO:23), the *prgI* gene of *Streptomyces alboniger* [Lacalle *et al.*, *EMBO J.*, 11:785 (1992)] (SEQ ID NO:24), and the *strS* gene of *Streptomyces griseus* [Distler *et al.*, *Gene*, 115:105 (1992)] (SEQ ID NO:25).

FIG. 5D illustrates the amino acid sequence identity between the sugar biosynthesis enzymes encoded by the *eryBV* and *eryCIII* genes of *Sac. erythraea* (SEQ ID NO:7 and SEQ ID NO:4 respectively) and the sugar biosynthesis enzyme encoded by the *dnrS* gene of *Streptomyces peucetius* [Otten *et al.*, *J. Bacteriol.*, 177:6688 (1995)] (SEQ ID NO:26).

FIG. 5E illustrates the amino acid sequence identity between the sugar biosynthesis enzyme encoded by the *eryCVI* gene of *Sac. erythraea* (SEQ ID NO:8) and the sugar biosynthesis enzymes encoded by the *smx* gene of *Streptomyces ambofaciens* [Geistlich *et al.*, *Mol. Microbiol.*, 6:2019 (1992)] (SEQ ID NO:27), the *rdmD* gene of *Streptomyces purpurascens* [GenBank Accession: U10405] (SEQ ID NO:28) and the glycine methyltransferase of *Rattus norvegicus* [Ogawa *et al.*, *Eur. J. Biochem.* 168:141 (1987)] (SEQ ID NO:29).

FIG. 6A through 6D illustrate the compounds conceivably formed in Examples 1-4 respectively and are representative of compounds formed from Type I (FIG. 6A), Type II (FIG. 6B), and Type III (FIGS. 6C and 6D) alterations.

FIG. 7 illustrates the construction of the expression plasmid pASX2 described in Example 2. For FIGS 7-13 the following abbreviations have been used: *amp*, ampicillin resistance gene; *tsr*, thiostrepton resistance gene; ROP, repressor of plasmid synthesis gene; *eryBI*, *eryBII*, *eryBIII*, *eryBIV*, *eryBV*, *eryBVI*, *eryBVII*, *eryCI*, *eryCII*, *eryCIII*, *eryCIV*, *eryCV*, and *eryCVI*, the erythromycin biosynthetic genes involved in the synthesis of mycarose or its attachment to the macrolide ring (*eryB*) or the synthesis of desosamine or its attachment to the macrolide ring (*eryC*) [the thin arrows above a gene indicate its relative size and the direction of transcription]; *ori-E. coli*, an origin of DNA replication that functions in *E. coli*, in the specific examples the ColE1 origin; *ori-Streptomyces*, an origin of DNA replication that functions in *Streptomyces*, in the specific examples the pJV1 origin [Servin-Gonzalez *et al.*, *Microbiology*, 141:2499 (1995)]; *p-ermE\**, a modified promoter for the erythromycin resistance gene; *t-fd*, the gene VIII transcription terminator of bacteriophage fd; PCR, polymerase chain reaction. Restriction enzyme sites have been indicated by their standard commercial names (i.e. *Bam*HI, *Eco*RI, etc). The abbreviations appended to the large arrows in the plasmid synthetic schemes summarize each of the steps involved the plasmid constructions. These steps are described fully in the relevant Examples.

FIG. 8 illustrates the construction of the *eryBVII* antisense expression plasmid pASBVII described in Example 2.



FIG. 9A illustrates the construction of the carrier plasmid pK1.

FIG. 9B-E illustrates the construction of plasmid pKB6 which carries all of the *eryB* genes and is described in Example 3.

FIG. 10 illustrates the construction of expression plasmid pX1 described in Example 3.

FIG. 11 illustrates the construction of the *eryB* expression plasmids pXSB6 and pXB6 described in Example 3.

FIG. 12A-B illustrate the construction of plasmid pKC4 which carries all of the *eryC* genes described in Example 4.

FIG. 13 illustrates the construction of the *eryC* expression plasmids pXSC4 and pXC4 described in Example 4.

### Detailed Description of the Invention

#### I. The Invention

The present invention provides isolated and purified polynucleotides that encode enzymes or fragments thereof responsible for the biosynthesis of polyketide-associated sugars or their attachment to polyketides, vectors containing those polynucleotides, host cells transformed with those vectors, a process of making novel glycosylated polyketides using those polynucleotides and vectors, and isolated and purified recombinant polypeptides and polypeptide fragments thereof.

#### II. Definitions

For the purposes of the present invention as disclosed and claimed herein, the following terms are defined.

The term "polyketide" as used herein refers to a large and diverse class of natural products, including but not limited to antibiotic, antifungal, anticancer, and anti-helminthic compounds. Antibiotics include, but are not limited to anthracyclines and macrolides of different types (polyenes and avermectins as well as classical macrolides such as erythromycins).

The term "glycosylated polyketide" refers to any polyketide that contains one or more sugar residues.

The term "glycosylation-modified polyketide" refers to a polyketide having a changed glycosylation pattern or configuration relative to that particular polyketide's unmodified or native state.

The term "polyketide-producing microorganism" as used herein includes any  
 5 microorganism that can produce a polyketide naturally or after being suitably engineered (i.e. genetically). Examples of actinomycetes and the polyketides they naturally produce include but are not limited to those listed in Table 1 below (see Hopwood, D.A. and Sherman, D.H., *Annu. Rev. Genet.*, 24:37-66 (1990) incorporated herein by reference).

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Table 1

Organism	Polyketide Produced
<i>Saccharopolyspora erythraea</i>	Erythromycin
<i>Streptomyces ambofaciens</i>	Spiramycin
<i>Streptomyces avermitilis</i>	Avermectin
<i>Streptomyces fradiae</i>	Tylosin
<i>Streptomyces griseus</i>	Candididin, monactin, griseusin
<i>Streptomyces violaceoniger</i>	Granaticin
<i>Streptomyces thermotolerans</i>	Carbomycin
<i>Streptomyces rimosus</i>	Oxytetracycline
<i>Streptomyces peucetius</i>	Daunorubicin
<i>Streptomyces coelicolor</i>	Actinorhodin
<i>Streptomyces glaucescens</i>	Tetracenomycin
<i>Streptomyces roseofulvus</i>	Frenolicin
<i>Streptomyces cinnamomensis</i>	Monensin
<i>Streptomyces curacoi</i>	Curamycin
<i>Amycolatopsis mediterranei</i>	Rifamycin

Other examples of polyketide-producing microorganisms that produce polyketides naturally include various *Actinomadura*, *Dactylosporangium* and *Nocardia* strains.

The term "sugar biosynthesis genes" as used herein refers to sequences of DNA from  
 15 *Saccharopolyspora erythraea* that encode sugar biosynthesis enzymes and is intended to include sequences of DNA from other polyketide-producing microorganisms which are identical or analogous to those obtained from *Saccharopolyspora erythraea*.

The term "sugar biosynthesis enzymes" as used herein refers to polypeptides which are involved in the biosynthesis and/or attachment of polyketide-associated sugars and their  
 20 derivatives and intermediates.

The term "polyketide-associated sugar" refers to a sugar that is known to attach to polyketides or that can be attached to polyketides by the processes described herein.

The term "sugar derivative" refers to a sugar which is naturally associated with a polyketide but which is altered relative to the unmodified or native state; examples only  
5 include N-3- $\alpha$ -desdimethyl D-desosamine, D-mycarose, 4-keto-L-mycarose, 4-keto-D-mycarose, 3-desmethyl L-mycarose and 3-desmethyl D-mycarose.

The term "sugar intermediate" refers to an intermediate compound produced in a sugar biosynthesis pathway.

The term "*eryB*" as used herein refers to sequences of DNA that encode enzymes  
10 involved specifically in the biosynthesis of the deoxysugar L-mycarose.

The term "*eryC*" as used herein refers to sequences of DNA that encode enzymes involved specifically in the biosynthesis of the deoxysugar D-desosamine.

### III. Polynucleotides

15 The organization of the segment of the *Saccharopolyspora erythraea* (*Sac. erythraea*) chromosome that determines the biosynthesis of erythromycin and the corresponding genes that determine the biosynthesis of the sugars L-mycarose and D-desosamine, designated *eryB* and *eryC*, respectively, are shown in FIG. 1A. It is seen that several genes are required for the biosynthesis of each of the sugars and that these genes are interspersed among one  
20 another. It is predicted that each gene encodes an enzyme that catalyzes one or a few steps in the biosynthesis of L-mycarose or D-desosamine from thymidine diphospho-4-keto-6 deoxyglucose (TDP-glucose); these steps are outlined in FIG. 2 and FIG. 3. In the case of L-mycarose, (shown in FIG. 2), these steps include: (1) C-2" deoxygenation, (2) C-2"/C-3" enoyl reduction, (3) C-5" epimerization, (4) C-3" C-methylation, (5) C-4" keto reduction, and  
25 (6) transfer to erythronolide B. For D-desosamine, shown in FIG. 3, these steps comprise (1) C-4'/3' isomerization, (2, 3) C-3' deoxygenation and reduction, (4) C-3' amination, (5, 6) N-3a' N-dimethylation, and transfer to mycarosyl erythronolide B.

This classification of genes (as belonging to either the *eryB* class or *eryC* class) was determined by first altering the wild type genes of interest in an erythromycin producing  
30 strain (i.e. *in vivo*) to inactivate their expression. The erythromycin products resulting from such alterations were then analyzed. Genes whose alterations caused an accumulation of erythronolide B (indicating a lack of L-mycarose, or failure to attach L-mycarose to the erythronolide ring) were classified as *eryB* genes; genes whose alterations caused an accumulation of 3- $\alpha$ -L-mycarosyl erythronolide B (indicating a lack of D-desosamine, or  
35 failure to attach D-desosamine to the 3- $\alpha$ -L-mycarosyl erythronolide B ring) were classified as *eryC* genes. Accordingly, it should be noted that all such genes identified herein as *eryB* or *eryC* are involved in the synthesis of L-mycarose or D-desosamine. The predicted

functional activities of the polypeptides encoded by *eryB* and *eryC* will be discussed in further detail below.

In one aspect then, the present invention provides isolated and purified *eryB* and *eryC* polynucleotides from *Sac. erythraea* that encode enzymes involved in the production of glycosylated polyketides. A polynucleotide of the present invention that encodes a sugar biosynthesis enzyme is an isolated single or double stranded polynucleotide having a nucleotide sequence which comprises (a) a nucleotide sequence selected from the group consisting of (i) the sense sequence of FIG. 4A (SEQ ID NO:1) from about nucleotide position 54 to about nucleotide position 1136; (ii) the sense sequence of SEQ ID NO:1 from about nucleotide position 1147 to about nucleotide position 2412; (iii) the sense sequence of SEQ ID NO:1 from about nucleotide position 2409 to about nucleotide position 3410; (iv) the sense sequence of FIG. 4B (SEQ ID NO:2) from about nucleotide position 80 to about nucleotide position 1048; (v) the sense sequence of SEQ ID NO:2 from about nucleotide position 1048 to about nucleotide position 2295; (vi) the sense sequence of SEQ ID NO:2 from about nucleotide position 2348 to about nucleotide position 3061; (vii) the sense sequence of SEQ ID NO:2 from about nucleotide position 3214 to about nucleotide position 4677; (viii) the sense sequence of SEQ ID NO:2 from about nucleotide position 4674 to about nucleotide position 5879; (ix) the sense sequence of SEQ ID NO:2 from about nucleotide position 5917 to about nucleotide position 7386; and (x) the sense sequence of SEQ ID NO:2 from about nucleotide position 7415 to about nucleotide position 7996;

- (b) sequences complementary to the sequences of (a),
- (c) sequences that, when expressed, encode polypeptides encoded by the sequences of (a), and
- (d) analogous sequences that hybridize under stringent conditions to the sequences of (a).

A preferred polynucleotide is a DNA molecule. In another embodiment, the polynucleotide is an RNA molecule.

The nucleotide sequence and deduced amino acid residue sequences of the sugar biosynthesis genes are set forth in FIG. 4A(1-4) and FIG. 4B(1-9). The nucleotide sequences of FIG. 4A(1-4) (SEQ ID NO:1) and FIG. 4B(1-9) (SEQ ID NO:2) represent full length DNA clones of the sense strand of two distinct clusters of sugar biosynthesis genes and are intended to represent both the sense strand (shown on top) and its complement. The amino acid sequences depicted below the sense strand correspond to polypeptides encoded by a nucleotide sequence selected from the group consisting of (i) the sense strand of SEQ ID NO:1 from about nucleotide position 54 to about nucleotide position 1136 (ii) the sense sequence of SEQ ID NO:1 from about nucleotide position 1147 to about nucleotide position 2412, (iii) the sense sequence of SEQ ID NO:1 from about nucleotide position 2409 to about

nucleotide position 3410, (iv) the sense sequence of SEQ ID NO:2 from about nucleotide position 80 to about nucleotide position 1048, (v) the sense sequence of SEQ ID NO:2 from about nucleotide position 1048 to about nucleotide position 2295, (vi) the sense sequence of SEQ ID NO:2 from about nucleotide position 2348 to about nucleotide position 3061, (vii) the sense sequence of SEQ ID NO:2 from about nucleotide position 3214 to about nucleotide position 4677, (ix) the sense sequence of SEQ ID NO:2 from about nucleotide position 5917 to about nucleotide position 7386 and (x) the sense sequence of SEQ ID NO:2 from about nucleotide position 7415 to about nucleotide position 7996. The polypeptides encoded by the nucleotide sequences of (i)-(x) above are set forth as SEQ ID NO:3-SEQ ID NO:12 respectively.

The present invention also contemplates analogous DNA sequences which hybridize under stringent hybridization conditions to the DNA sequences set forth above. Stringent hybridization conditions are well known in the art and define a degree of sequence identity greater than about 80%-90%. The modifier "analogous" refers to those nucleotide sequences that encode analogous polypeptides (i.e. in relation to a sugar biosynthesis enzyme), analogous polypeptides being those which have only conservative differences and which retain the conventional characteristics and activities of sugar biosynthesis enzymes. (A more detailed description of analogous polypeptides is provided below). The present invention also contemplates naturally occurring allelic variations and mutations of the DNA sequences set forth above so long as those variations and mutations code, on expression, for a sugar biosynthesis gene of this invention as set forth hereinafter.

As is well known in the art, because of the degeneracy of the genetic code, there are numerous other DNA and RNA molecules that can code for the same polypeptides as those encoded by the aforementioned sugar biosynthesis genes and fragments thereof. The present invention, therefore, contemplates those other DNA and RNA molecules which, on expression, encode the polypeptides of SEQ ID NO:3-SEQ ID NO:11 or fragments thereof. Having identified the amino acid residue sequence encoded by a sugar biosynthesis gene, and with knowledge of all triplet codons for each particular amino acid residue, it is possible to describe all such encoding RNA and DNA sequences. DNA and RNA molecules other than those specifically disclosed herein and, which molecules are characterized simply by a change in a codon for a particular amino acid, are within the scope of this invention.

The 20 common amino acids and their representative abbreviations, symbols and codons are well known in the art (see for example, *Molecular Biology of the Cell*, Second Edition, B. Alberts *et al.*, Garland Publishing Inc., New York and London, 1989). As is also well known in the art, codons constitute triplet sequences of nucleotides in mRNA molecules and as such, are characterized by the base uracil (U) in place of base thymidine (T) which is present in DNA molecules. A simple change in a codon for the same amino acid residue

within a polynucleotide will not change the structure of the encoded polypeptide. By way of example, it can be seen from SEQ ID NO:1 that an AGC codon for serine exists at nucleotide positions 126-128 and again at positions 420-422 and 561-563. However, it can also be seen from that same sequence that serine can be encoded by a TCG codon (see eg. nucleotide  
5 positions 192-194) and a TCC codon (see e.g., nucleotide positions 204-206). Substitution of the latter codons for serine with the AGC codon for serine, or *visa versa*, does not substantially alter the DNA sequence of SEQ ID NO:1 and results in production of the same polypeptide. In a similar manner, substitutions of the recited codons with other equivalent codons can be made in a like manner without departing from the scope of the present  
10 invention.

A polynucleotide of the present invention can also be an RNA molecule. An RNA molecule contemplated by the present invention is complementary to or hybridizes under stringent conditions to any of the DNA sequences set forth above. Exemplary and preferred RNA molecules are mRNA molecules that encode sugar biosynthesis enzymes of this  
15 invention.

#### IV. Polypeptides

In another aspect, the present invention provides polypeptides which are reasonably believed to be sugar biosynthesis enzymes. A sugar biosynthesis enzyme of the present  
20 invention is a polypeptide of about 21 kdal to about 47 kdal. As set forth in FIG. 5A-5E, analogs of the predicted polypeptides encoded by certain *eryB* and *eryC* genes have been identified in various species and their sequences compared using the PRETTY routine (Genetics Computer Group (GCG) Sequence Analysis Software Package, Madison, WI). Due to the degree of amino acid sequence identity existing between the polypeptides of these  
25 other sugar biosynthesis genes and the polypeptides encoded by the *eryB* and *eryC* genes, certain enzymatic activities can reasonably be attributed to the *eryB* and *eryC* polypeptides.

By way of example, analogs of the polypeptide encoded by the *eryBIV* gene have been identified in *Yersinia pseudotuberculosis*, *Salmonella enterica*, *Streptomyces griseus* and *Escherichia coli* (see FIG. 5A). The various analogs have been identified with from 290-328  
30 amino acid residues and are characterized by a low degree of amino acid sequence identity. (For example, the identity between the sugar biosynthesis enzyme encoded by the *eryBIV* gene of *Sac. erythraea* and the sugar biosynthesis enzyme encoded by the *galE* gene of *E. coli* is 20% at the amino acid level). However, a conserved amino acid sequence motif, G x x G x x G (where G represents the amino acid glycine and x represents any other amino acid  
35 residue) is found within the first 30 amino acid residues of all analogs shown. Since the polypeptide encoded by the *galE* gene has been shown to be an epimerase (whose mechanism includes a ketoreduction (Bauer *et al.*, *Proteins* 12:372 (1992))), the *eryBIV* gene product is

reasonably predicted to be a ketoreductase.

As set forth in FIG. 5B analogs of the sugar biosynthesis enzyme encoded by the *eryBVII* gene have been identified in *Streptomyces griseus*, *Salmonella enterica*, *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. The various analogs have been identified with  
5 from 183-200 amino acid residues and are characterized by a moderate degree of amino acid identity. By way of example, the identity at the amino acid level between the sugar biosynthesis enzyme encoded by the *eryBVII* gene of *Sac. erythraea* and the sugar biosynthesis enzyme encoded by the *rfbC* gene of *Salmonella enterica* or the *strM* gene of *Streptomyces griseus* is 37% and 61%, respectively. Furthermore, a common characteristic  
10 of these particular polypeptides (including that of *eryBVII*), is that they are only associated with L-sugar biosynthesis and not with D-sugar biosynthesis. Thus the gene product of *eryBVII* is reasonably predicted to function as a C-5 epimerase which converts the stereochemistry of the sugar from the "D" configuration to the "L" configuration.

As set forth in FIG. 5C analogs of the sugar biosynthesis enzyme encoded by the  
15 *eryCIV* gene have been identified in *Sac. erythraea* and *Yersinia pseudotuberculosis*. As set forth in FIG. 5C, the predicted amino acid sequences of the protein products of *eryCI* and *eryCIV* share 34% sequence identity to each other, 27% and 25% respectively to the predicted amino acid sequence encoded by *ascC* from *Yersinia pseudotuberculosis*. The enzyme encoded by *ascC* has been shown to remove a hydroxyl group located at the C-3  
20 position of L-ascarylose (Liu and Thorson, *Annu. Rev. Microbiol.* 48:223 (1994)). Thus, at least one of the polypeptides encoded by *eryCI* or *eryCIV* is predicted to be an enzyme which functions in deoxygenation reactions.

Furthermore, the enzyme encoded by the *ascC* gene requires the biochemical cofactor pyridoxamine, which is the same cofactor used in biochemical transamination reactions.  
25 Consequently, it has been proposed that some protein analogs (such as *dnrJ* from *Streptomyces peucetius*, *prg1* from *Streptomyces alboniger* and *strs* from *Streptomyces griseus*) having a moderate degree of sequence similarity to the polypeptide encoded by *ascC* function as transaminases in amino sugar biosynthesis (Thorson *et al.*, *J. Am. Chem. Soc.* 115:6993 (1993)). Since the biosynthesis of D-desosamine requires both deoxygenation and  
30 transamination, it is reasonable to predict that at least one of the polypeptides encoded by the *eryCI* or *eryCIV* genes functions in transamination reactions.

As set forth in FIG. 5D the predicted polypeptides encoded by *eryBV* and *eryCIII* share 43% identity at the amino acid level and as such, may be assumed to have similar activities with respect to their particular sugars. However, as shown in FIGS. 2 and 3, there  
35 are no common steps in the proposed pathways of L-mycarose and D-desosamine biosynthesis. Rather than having similar sugar biosynthesis functions, these polypeptides are predicted to be nucleotidyl-sugar transferases which, (in *Sac. erythraea* at least), function to

attach L-mycarose and D-desosamine to erythronolide B and 3- $\alpha$ -mycarosylerythronolide B, respectively.

As set forth in FIG. 5E analogs of the polypeptide encoded by the *eryCVI* gene have been identified in *Streptomyces ambofaciens*, *Streptomyces purpurascens*, and *Rattus norvegicus*. The various analogs have been identified with from 237-293 amino acid residues and are characterized by a low to moderate degree of amino acid identity. By way of example, the identity between the polypeptide encoded by the *eryCVI* gene of *Sac. erythraea* and the glycine methyltransferase of *Rattus norvegicus* is 26% at the amino acid level. Furthermore these sugar biosynthesis enzymes share a common sequence motif, LDVACGTG (SEQ ID NO:30 = amino acid positions 64-71 in the consensus sequence in FIG. 5E), with rat glycine methyltransferase whose biochemical function is known (Ogawa *et al.*, *Eur. J. Biochem.* 168:141 (1987)). Thus these polypeptides are predicted to be N-methyltransferases.

In another aspect, the present invention provides a recombinant C-4" keto reductase from *Sac. erythraea*. A recombinant *Sac. erythraea* C-4" ketoreductase of the present invention is a polypeptide of about 322 or less amino acid residues. A preferred recombinant *Sac. erythraea* C-4" ketoreductase is that encoded by the nucleotide sequence of SEQ ID NO:2 from about nucleotide position 80 to about nucleotide position 1048.

The present invention also contemplates amino acid residue sequences that are substantially duplicative of the sequences set forth herein such that those sequences demonstrate like biological activity to disclosed sequences. Such contemplated sequences include those analogous sequences characterized by a minimal change in amino acid residue sequence or type (e.g., conservatively substituted sequences) which insubstantial change does not alter the fundamental nature and biological activity of the aforementioned sugar biosynthesis enzymes.

It is well known in the art that modifications and changes can be made in the structure of a polypeptide without substantially altering the biological function of that peptide. For example, certain amino acids can be substituted for other amino acids in a given polypeptide without any appreciable loss of function. In making such changes, substitutions of like amino acid residues can be made on the basis of relative similarity of side-chain substituents, for example, their size, charge, hydrophobicity, hydrophilicity, and the like.

As detailed in United States Patent No. 4,554,101, incorporated herein by reference, the following hydrophilicity values have been assigned to amino acid residues: Arg (+3.0); Lys (+3.0); Asp (+3.0); Glu (+3.0); Ser (+0.3); Asn (+0.2); Gln (+0.2); Gly (0); Pro (-0.5); Thr (-0.4); Ala (-0.5); His (-0.5); Cys (-1.0); Met (-1.3); Val (-1.5); Leu (-1.8); Ile (-1.8); Tyr (-2.3); Phe (-2.5); and Trp (-3.4). It is understood that an amino acid residue can be substituted for another having a similar hydrophilicity value (e.g., within a value of plus or



minus 2.0) and still obtain a biologically equivalent polypeptide.

In a similar manner, substitutions can be made on the basis of similarity in hydropathic index. Each amino acid residue has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics. Those hydropathic index values are:

5 Ile (+4.5); Val (+4.2); Leu (+3.8); Phe (+2.8); Cys (+2.5); Met (+1.9); Ala (+1.8); Gly (-0.4); Thr (-0.7); Ser (-0.8); Trp (-0.9); Tyr (-1.3); Pro (-1.6); His (-3.2); Glu (-3.5); Gln (-3.5); Asp (-3.5); Asn (-3.5); Lys (-3.9); and Arg (-4.5). In making a substitution based on the hydropathic index, a value of within plus or minus 2.0 is preferred.

10 **V. Production of novel glycosylated polyketides**

In another aspect, the present invention comprises a general procedure for producing novel polyketide structures *in vivo* by selectively altering, inactivating, or augmenting the genetic information of the organism that naturally produces a related polyketide. That is, in the present invention, novel polyketides of desired structure are produced by manipulation of

15 the *eryB* and/or *eryC* genes followed by their introduction into various polyketide-producing microorganisms. These manipulations result in the formation of "glycosylation-modified" polyketides (i.e. polyketides having an altered glycosylation pattern or configuration relative to their native state). For example, "glycosylation-modified" polyketides are those which have additional sugar groups attached (where none previously existed), different sugars (such

20 as sugar intermediates) attached in place of the natural sugars or lack sugar groups (at positions where sugar groups previously existed).

In the case of Type I and Type II alterations (further described below) glycosylation-modified polyketides may arise through mechanisms which cause either (1) the non-production of the sugar attachment enzyme (i.e. the enzyme involved in attachment of a sugar

25 to the polyketide structure) or (2) the non-production of a sugar biosynthesis enzyme. In the first instance, the sugar will not be attached to the polyketide since the enzyme which functions to attach the sugar will be lacking. In the second situation, a sugar intermediate from the biosynthesis pathway will be produced (depending on which enzyme is lacking) and attached to the polyketide provided it is recognized as a suitable substrate by the sugar

30 attachment enzyme; alternatively, it will not be recognized and therefore, not attached. In the case of Type III alterations (also described in detail below), glycosylation-modified polyketides arise via attachment of additional or different sugars (i.e. not normally found in a particular polyketide-producing strain) to the polyketide. It should be noted, that these postulated mechanisms are simply provided to enhance understanding of the novel processes described herein; the actual mechanisms by which the Type I, II and III alterations produce

35 glycosylation-modified polyketides is not presently known.

In the first type of alteration (referred to herein as Type I alterations), genetically

altered *eryB* and/or *eryC* genes are introduced into the chromosome of *Sac. erythraea* or another glycosylated polyketide-producing organism that also produces L-mycarose, D-desosamine, or their closely related derivatives such as mycaminose (4-hydroxy D-desosamine). The genetic alteration of an *eryB* and/or *eryC* gene is such that it causes a non-functional enzyme to be synthesized. Once introduced into an appropriate strain, the altered gene replaces its corresponding wild type gene causing the strain to lose the ability to produce a particular enzymatic activity involved in sugar biosynthesis. As a result, a glycosylation-modified polyketide is produced via either of the mechanisms previously described for a Type I alteration.

In a Type I change described herein, a specific mutation in an *eryB* and/or *eryC* gene of the *Sac. erythraea* chromosome is accomplished by a three step process which involves: 1) specifically altering the DNA sequence of a desired sugar biosynthesis gene, 2) subcloning the altered sequence into a suitable vector capable of recombining in the chromosome of an appropriate host and 3) introducing the vector containing the subcloned sequence into the appropriate host so that exchange of the wild type allele with the mutated one will occur. The first step is accomplished using standard recombinant DNA techniques to effect a deletion, base pair conversion or frame-shift in the DNA sequence. The second step, which also employs standard recombinant techniques, involves subcloning the altered sequence into a vector which does not replicate in *Sac. erythraea* or the desired host. In the final step, the vector is introduced into a suitable host, where by the process of gene replacement, the altered allele replaces the wild-type one. All techniques employed in a Type I change are well known to those of ordinary skill in the art.

Example 1 illustrates the process of gene replacement of an *eryB* gene. As Example 1 shows, the *eryB* gene of interest is mutated and along with adjacent upstream and downstream DNA sequences, cloned into a non-replicating *Sac. erythraea* plasmid vector. The vector carrying the mutated allele and adjoining DNA is then introduced into the host strain by the process of protoplast transformation. Transformants are regenerated under selective conditions (i.e. conditions that require expression of a particular plasmid marker) in order to induce recombination of the plasmid into the host cell chromosome. In other words, since the plasmid does not replicate autonomously, it must reside in the chromosome to be maintained in the cell and to express a particular marker under selective conditions. Insertion is achieved when the regenerated cells undergo a single homologous recombination between one of the two DNA segments that flank the mutation on the plasmid and its homologous counterpart in the chromosome. The cells are then grown without selection for the marker which induces plasmid loss from the chromosome. This loss arises after the cells have undergone a second recombination between the second DNA segment that flanks the mutation and its homologous chromosomal counterpart. This second recombinational event

results in the loss of the plasmid sequences and the wild type allele from the chromosome; the mutant allele however is retained.

In a variation of a Type I change, the non-production of the sugar biosynthesis enzyme (or attachment enzyme) may be achieved by the alternative mechanisms of promoter inactivation and/or transcriptional terminator insertion. These variations do not effect the gene sequence itself but rather regulatory mechanisms involved in gene transcription. "Promoter" as used herein refers to that region of a DNA molecule which controls the initiation of RNA transcription. Such regions are known to bind RNA polymerases (i.e. the enzymes involved in synthesizing RNA molecules). This form of Type I change (i.e. promoter inactivation) involves two steps of 1) identifying the promoter region of the desired gene and 2) rendering the promoter region inoperable by mutation. As in the replacement mechanism described above such mutations may be effected by creating deletions in the promoter sequence or by base pair conversion. In the case where the promoter controls transcription of a single gene, inactivation of the promoter will eliminate expression of that particular gene; of course, where the promoter controls expression of an entire operon (i.e. a series of genes whose expression is controlled by a single promoter), promoter inactivation will effectively eliminate expression of all genes in that operon.

In a similar manner, the non-production of a sugar biosynthesis enzyme (or attachment enzyme) may arise from inserting a transcriptional terminator upstream from the gene to be inactivated. A "transcriptional terminator" as used herein is a nucleotide sequence which signals RNA polymerase to cease transcription. An example of a transcriptional terminator is a palindromic sequence capable of forming a stem-loop structure that is followed by a stretch of U residues (for example the transcriptional terminator that follows gene VIII of bacteriophage fd (Beck and Zink, *Gene*, 16:35 (1981))). Effecting a change in production of a sugar biosynthesis gene by this process involves 1) identifying of the gene or genes of interest (in the case of an operon arrangement) to be inactivated and 2) cloning a transcriptional terminator sequence in a region of the DNA upstream from such gene(s). A transcriptional terminator will cause the polymerase involved in RNA transcription to stop (at or near the signaling region) thereby preventing transcription of any downstream sequences. Thus, changes such as promoter inactivation and transcriptional insertion, which directly effect expression of sugar biosynthesis genes are also intended to be within the scope of the invention.

In the second case (referred to herein as Type II alterations) *eryB* and/or *eryC* genes are arranged on a vector in an antisense orientation relative to a promoter capable of allowing expression of the gene in *Sac. erythraea* or *Streptomyces*. The vector is then introduced into a polyketide producing microorganism. As a result of this vector construction, antisense messenger RNA (mRNA) is produced which interferes with the translation of the wild-type

mRNA. Similarly to the Type I manipulation, novel glycosylation modified polyketides will be produced in which the normal mycarose, desosamine, and/or closely related sugar residue is lacking or is substituted by a sugar intermediate.

In a Type II change, inactivation of the *eryB* and/or *eryC* genes by antisense expression is accomplished by a two step procedure in which (1) a specific sugar biosynthesis gene is subcloned into an expression vector in an antisense (i.e. reverse) orientation; and (2) the anti-sense expression vector is introduced into the desired strain. The first step is accomplished using standard recombinant DNA techniques employing either *E. coli* or *Streptomyces* as the host, and an expression vector (capable of replicating in either host) that can be assembled to contain a *Streptomyces* promoter. *Streptomyces* promoters may be obtained from any commercially available *Streptomyces* plasmids or *Streptomyces-E. coli* shuttle plasmids. In step 2, the anti-sense expression vector is introduced into a suitable *Streptomyces* strain and the transformed cells are grown under selective conditions in order to maintain the expression plasmid in the cell.

As described in Example 2, the gene to be inactivated is subcloned in its reverse orientation downstream of a *Streptomyces* promoter (which is contained within a replicating *Sac. erythraea* plasmid). The plasmid carrying the antisense gene is then introduced into the host strain by protoplast transformation. Transformants are regenerated under selective conditions in order to maintain the autonomously replicating plasmid in the cells. Subsequent expression of the antisense gene causes the production of an antisense messenger RNA (mRNA) that is complementary to the mRNA of the native allele of the selected gene. Through standard nucleotide base pair interactions, the antisense mRNA and the native mRNA form an RNA duplex that occludes the ribosome binding site of the native mRNA. This interaction prevents ribosomal translation of the native mRNA and the corresponding synthesis of the enzyme encoded by that mRNA. In this way, specific enzymatic steps in sugar biosynthesis corresponding to the identity of the gene expressed in the antisense orientation are blocked leading to the production of novel sugar intermediates which, when attached to the polyketide ring of the host microorganism, give rise to novel glycosylation-modified polyketides. Alternatively, the antisense expression vector can be constructed using a non-replicating *Sac. erythraea* vector that includes flanking DNA from a nonessential region of the *Sac. erythraea* chromosome, such as the region immediately upstream from the *eryK* gene (FIG. 1). This vector can then be used to stably insert the antisense construction into the chromosome by homologous recombination in a fashion similar to that described for the construction of a Type I alteration.

In the third case (referred to herein as Type III alterations), novel glycosylation-modified polyketides of desired structure are produced by arranging all or a subset of the *eryB* and/or *eryC* genes on a replicating vector and introducing these genes *en bloc* into a

“distinct” polyketide-producing organism, ie. one other than the microorganism from which the *eryB* and/or *eryC* genes were taken. As an example, *eryB* and/or *eryC* genes may be taken from *Sac. erythraea* and introduced into *Streptomyces violaceoniger* or *Streptomyces venezuelae*. In this case, mycarose, desosamine, their biochemical intermediates and/or their  
5 closely related derivatives will be synthesized and attached at specific positions to polyketide compounds that do not necessarily carry these, or any, sugar residues. Some examples of novel glycosylated polyketides that may be produced in hosts that carry such manipulations are shown in FIG. 6.

In Type III changes, the genes for the biosynthesis of mycarose and/or desosamine are  
10 introduced into a polyketide-producing organism other than *Sac. erythraea* by another simple two step procedure: 1) all or a subset of the *eryB* and/or *eryC* genes are assembled together on a replicating plasmid downstream of a *Streptomyces* promoter; and 2) the plasmid is introduced into the polyketide-producing organism. Step 1 requires standard recombinant DNA manipulations employing *E. coli* and/or *Streptomyces* as the host. Step 2 requires one  
15 or more plasmids out of the several *Streptomyces* vectors or *E. coli-Streptomyces* shuttle vectors available, one or more promoters that function in *Streptomyces*, and a selection for the presence of the strain carrying the plasmid. As described in Examples 3 and 4, sets of the *eryB* and/or *eryC* genes are sequentially subcloned together on a replicating vector downstream of a suitable promoter that functions in the desired host. The plasmid carrying  
20 the grouped genes is then introduced into the host strain by electroporation or by transformation of protoplasts employing selection for a plasmid marker.

## GENERAL METHODS

### 25 Materials, Plasmids, and Bacterial Strains

Restriction endonucleases, T4 DNA ligase, competent *E. coli* DH5 $\alpha$  cells, X-gal, IPTG and plasmids pUC18, pUC19, and pBR322 were purchased from Bethesda Research Laboratories (BRL), Gaithersburg, MD. Vent $\alpha$ <sup>®</sup> DNA polymerase was purchased from New  
30 England Biolabs (Beverly, MA). Plasmids pGEM $\alpha$ 5Zf, pGEM $\alpha$ 7Zf, and pGEM $\alpha$ 11Zf were from Promega, Madison, WI, plasmids pIJ4070 and pIJ702 were obtained from the John Innes Institute, Norwich, England, and plasmids pWHM3 and pWHM4 (*J. Bacteriol.* 1989 171:5872) were obtained from C. R. Hutchinson, University of Wisconsin, Madison, WI. [ $\alpha$ -<sup>32</sup>P]dCTP, Hybond<sup>™</sup>-N nylon membranes, and Megaprime nick translation kits were  
35 from Amersham Corp., Chicago, IL. SeaKem $\alpha$  LE agarose and SeaPlaque $\alpha$  low gelling temperature agarose were from FMC Bioproducts, Rockland, ME. *E. coli* K12 strains carrying the *E. coli-Sac. erythraea* shuttle plasmids pWHM3 and pWHM4 (Vara *et al.*, *J*

*Bacteriol.*, 171:5872 (1989)) and pAIX have been deposited at the Agricultural Research Culture Collection (NRRL) 1815 N. University Street, Peoria, Illinois 61604, as of December 5, 1995, under the terms of the Budapest Treaty and will be maintained for a period of thirty (30) years from the date of deposit, or for five (5) years after the last request  
5 for the deposit, or for the enforceable period of the U.S. patent, whichever is longer. Plasmids pWHM3, pWHM4 and pAIX were accorded the accession numbers NRRL B-21512, NRRL B-21513 and NRRL B-21514, respectively. *Sac. erythraea* strain NRRL2338 is also available from the Agricultural Research Service culture collection. *Staphylococcus aureus* Th<sup>R</sup> (thiostrepton resistant) was obtained by plating 10<sup>8</sup> cells of *S. aureus* on agar  
10 medium containing 10 µg/ml thiostrepton and picking a survivor after 48 hr growth at 37°C. Thiostrepton was obtained from Sigma Chemical, St. Louis, MO. All other chemicals and reagents were from standard commercial sources unless otherwise specified.

#### DNA Manipulations

15 Standard conditions were employed for restriction endonuclease digestion, agarose gel-electrophoresis, isolation of DNA fragments from low melting agarose gels, DNA ligation, plasmid isolation from *E. coli* by alkaline lysis, and transformation of *E. coli* employing selection for ampicillin resistance (150 µg/ml) on LB agar plates (Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Plainview,  
20 NY, 1989). Total DNA from *Sac. erythraea* and *Streptomyces* species (including *S. fradiae*, *S. celestes*, *S. violaceoniger*, *S. hygroscopicus*, *S. venezuelae*) was prepared according to described procedures (Hopwood *et al.*, *Genetic Manipulation of Streptomyces, A Laboratory Manual*, John Innes Foundation, Norwich, UK (1985)). Transfer of DNA from agarose gels to Hybond<sup>TM</sup>-N membranes and Southern analysis using Megaprime<sup>TM</sup> nick translated probes  
25 was performed according to the manufacturers instructions.

#### Amplification of DNA Fragments

Synthetic deoxyoligonucleotides were synthesized on an ABI Model 380A synthesizer (Applied Biosystems, Foster City, CA) following the manufacturers  
30 recommendations. Amplification of DNA fragments was performed by the polymerase chain reaction (PCR) using a Perkin Elmer GeneAmp<sup>®</sup> PCR System 9600. Reactions contained 100 pmol of each primer, 1 µg of template DNA (chromosomal DNA from *Sac. erythraea* NRRL2338), 2 units Vent<sup>R</sup> DNA polymerase in 100 µl volume of PCR buffer (10 mM KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 mM Tris-HCl (pH 8.8, @ 25°C), 2.5 mM MgSO<sub>4</sub>, 0.1% Triton<sup>®</sup> X-  
35 100) containing dATP (200 µM), dTTP (200 µM), dCTP (250 µM), and dGTP (250 µM). The reaction mixture was subjected to 30 cycles. Each cycle consisted of one period of 35 sec at 96°C and one period of 2 min at 72°C. The reaction products were visualized and

purified from low melting agarose. The PCR primers described in the examples were derived from the nucleotide sequence of the *eryB* and *eryC* genes of FIG. 4.

#### Transformation and Gene Replacement in *Sac. erythraea*

5        Protoplasts of *Sac. erythraea* strains were prepared and transformed with miniprep DNA isolated from *E. coli* according to published procedures (Yamamoto *et al.*, *J Antibiotics*, 39:1304 (1986)). Non-integrative transformants, in the case of pWHM4 derivatives, were selected by regenerating the protoplasts and overlaying with thiostrepton (final concentration 20 µg/ml) as described (Weber *et al.*, *Gene*, 68:173 (1988)). Integrative  
10        transformants, in the case of pWHM3 derivatives, were selected on thiostrepton-containing agar plates (15 µg/ml) as described by Weber *et al.*, *Gene*, 68:173 (1988). Loss of the Th<sup>R</sup> phenotype was monitored after two rounds of non-selective growth in SGGP media (Yamamoto *et al.*, *J Antibiotics*, 39:1304 (1986)) followed by protoplasting and serial  
15        dilution on non-selective agar media. Regenerated protoplasts were replica plated on thiostrepton-containing media. Th<sup>S</sup> (thiostrepton-sensitive) colonies arose at a frequency of 10<sup>-1</sup>. Retention of the mutant allele was established by Southern hybridization of several Th<sup>S</sup> colonies.

#### Fermentation

20        *Sac. erythraea* or *Streptomyces* cells are inoculated into 100 ml SCM medium (1.5% soluble starch, 2.0% Difco Soytone, 0.15% Yeast Extract, 0.01% CaCl<sub>2</sub>) and allowed to grow for 3 to 6 days. The entire culture is then inoculated into 10 liters of fresh SCM medium. The fermenter is operated for a period of 4 to 7 days at 32°C maintaining constant aeration and pH at 7.0. After the fermentation is complete, the cells are removed by centrifugation at  
25        4°C and the fermentation beer is kept cold until further use. When antibiotic selection to maintain a plasmid, such as pXC4 or pXB6, is required, thiostrepton (10µg/ml) is added to both the 100 ml starter culture and the 10-liter fermenter.

30        The invention will be better understood in connection with the following examples, which are intended as an illustration of and not a limitation upon the scope of the invention. Both below and throughout the specification, it is intended that citations to the literature be expressly incorporated by reference.

#### Example 1: Construction and characterization of *Sac. erythraea* ERBIV that produces 4"-deoxy-4"-oxo-erythromycin A

35

A. Construction of Plasmid pRBIV: A 4.3 kb *Pst*I-*Hind*III fragment, which included

the *eryBIV* gene, was isolated from the plasmid pAIX5 and subcloned into *Pst*I-*Hind*III digested pUC19 to generate plasmid pUCBIV. After transformation and isolation of the plasmid from *E. coli*, the identity of pUCBIV was confirmed by digestion with *Mun*I which released a fragment of 370 bp. Plasmid pUCBIV was then cut with the restriction enzyme *Nco*I, the restriction site filled in with Klenow enzyme, and the plasmid religated to generate plasmid pNCOBIV, (which now carried a frameshift mutation in the *eryBIV* gene). After transformation and isolation of the plasmid from *E. coli*, the identity of pNCOBIV was confirmed by digestion with *Nsi*I and *Hind*III which released a fragment of 1.59 kb. (The *Nsi*I site was formed by the fill-in and religation of the *Nco*I site.) Finally, plasmid pNCOBIV was digested with *Hind*III and *Sst*I and the 3.2 kb fragment carrying the altered *eryBIV* gene was isolated and ligated into *Hind*III and *Sst*I digested pWHM3 to generate plasmid pRBIV. After transformation and isolation of the plasmid from *E. coli*, the identity of pRBIV was confirmed by digestion with *Kpn*I which released fragments of 5.2 kb, 4.4 kb, and 0.72 kb.

15        **B. Construction of *Sac. erythraea* ERBIV:** *Sac. erythraea* protoplasts were transformed with plasmid pRBIV and integrative transformants selected as described in General Methods. Resolution of the integrants by nonselective growth as described in General Methods yielded *Sac. erythraea* ERBIV in which the wild type copy of the *eryBIV* gene was replaced with the inactive mutant copy. Gene replacement was confirmed by Southern analysis of *Nco*I digested *Sac. erythraea* DNA and *Nco*I-*Nsi*I digested *Sac. erythraea* DNA using the 1.58 kb *Nco*I-*Hind*III fragment isolated from plasmid pUCBIV (coordinates 681-2214, FIG. 4B) as a probe. Wild type *Sac. erythraea* and wild type resolvants display a hybridizing DNA fragment of 2.75 kb when digested with either *Nco*I or *Nco*I-*Nsi*I, whereas *Sac. erythraea* strain ERBIV is characterized by hybridization to either a 16 kb DNA fragment or a 2.75 kb DNA fragment when digested with *Nco*I or *Nco*I-*Nsi*I, respectively.

30        **C. Isolation, purification, and properties of 4"-deoxy-4"-oxo-erythromycin A from *Sac. erythraea* ERBIV:** *Sac. erythraea* strain ERBIV is fermented for 4 days in SCM media as described in General Methods. The fermentation broth of *Sac. erythraea* ERBIV is then cooled to 4°C and adjusted to pH 4.0 and extracted once with methylene chloride. The aqueous layer is readjusted to pH 9.0 and extracted twice with methylene chloride and the combined basic methylene chloride extracts are concentrated to a solid residue. This is digested in methanol and chromatographed over a column of Sephadex LH-20 in methanol. Fractions are tested for bioactivity against a sensitive organism, such as *Staphylococcus aureus* Th<sup>R</sup>, and active fractions are combined. The combined fractions are concentrated and the residue is digested in 10 ml of the upper phase of a solvent system consisting of n-heptane, benzene, acetone, isopropanol, 0.05 M, pH 7.0 aqueous phosphate buffer



(5:10:3:2:5, v/v/v/v/v), and chromatographed on an Ito Coil Planet Centrifuge in the same system. Active fractions are combined, concentrated and partitioned between methylene chloride and dilute ammonium hydroxide (pH 9.0). The methylene chloride layer is separated and concentrated to yield the desired product as a white foam.

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Example 2: Construction and characterization of *Sac. erythraea* ER720(pASBVII) that produces 3- $\alpha$ -D-mycarosyl-5- $\beta$ -D-desosaminoyl-12-hydroxy-erythronolide B

A. Construction of plasmid pASX2 (see FIG. 7): The 290 bp *EcoRI*-*Bam*HI segment  
 10 carrying the *ermE*\* promoter is isolated from plasmid pIJ4070 and ligated into *EcoRI*-*Bam*HI  
 digested pWHM4 DNA to form pASX1. After transformation and isolation of the plasmid  
 from *E. coli*, the identity of pASX1 is confirmed by digestion with *Apa*LI which releases  
 fragments of 3.9 kb, 2.5 kb, 1.2 kb, 0.5 kb, and 0.4 kb. Two oligonucleotides of the  
 sequences: SEQ ID NO:31 (5'-GATCCAGCGTCTGCAGGCATGCTCTAGATACAATTA  
 15 AAGGCTCCTTTTGGAGCCTTTTTTTTTTGGAGATTTTCAACGT-3') and  
 SEQ ID NO:32 (5'-AGCTACGTTGAAAATCTCCAAAAAAAAGGCTCCAAAA  
 GGAGCCTTTAATTGTATCTAGAGCATGCCTGCAGACGCTG-3'), corresponding to the  
 (+) and (-) strands of the bacteriophage fd gene VIII transcription terminator (t-fd) (Beck *et*  
*al.* (1978) *Nucl. Acids Res.* 5:4495)] and including restriction enzyme sites for the enzymes  
 20 *Pst*I, *Sph*I, and *Xba*I, and overhanging ends compatible with *Bam*HI and *Hind*III are  
 synthesized and approximately 250 ng of each oligonucleotide are then mixed together in TE  
 buffer and heated to 99°C for 1 min. The solution is cooled slowly to room temperature  
 allowing the oligonucleotides to anneal due to self complementarity, and the annealed  
 oligonucleotides are then ligated into *Bam*HI-*Hind*III digested pASX1 to give pASX2. After  
 25 transformation and isolation of the plasmid from *E. coli*, the identity of pASX2 is confirmed  
 by DNA sequencing of the 1.2 kb *EcoRI*-*Sal*I fragment that contains the *ErmE*\* promoter and  
 the bacteriophage fd terminator.

B. Construction of plasmid pASBVII (see FIG. 8): The 598 base pair DNA segment  
 that carries the *eryBVII* gene, comprising coordinates 7398-7996 (FIG. 4B), is amplified by  
 30 PCR employing two oligonucleotides, SEQ ID NO:33 (5'-  
 GATCGCATGCTCTAGAGTACG-TGAGCTGGCGGTGGCGGGC-3') and SEQ ID NO:34  
 (5'-GATCCGGATCCGCATGCTT-CACCTGCCGGTGCTGGCGGG-3'). After digestion of  
 the purified PCR product with *Bam*HI-*Xba*I the PCR fragment was ligated to *Bam*HI-*Xba*I  
 digested pASX2 to give pASBVII. After transformation and isolation of the plasmid from *E.*  
 35 *coli*, the identity of pASBVII is verified by DNA sequencing of the 880 bp *EcoRI*-*Xba*I  
 insert.

C. Construction of *Sac. erythraea* ER720(pASBVII): *Sac. erythraea* strain ER720

protoplasts are transformed with plasmid pASBVII and transformants are selected for with thiostrepton (15 µg/ml). To confirm transformation, total DNA is isolated from Th<sup>R</sup> colonies and used to transform *E. coli*. After transformation and isolation of the plasmid from *E. coli*, the identity of pASBVII is verified by restriction analysis with the enzymes *Pvu*II and *Bam*HI which releases a 1.48 kb fragment. Those *Sac. erythraea* colonies that are found to contain pASBVII are designated *Sac. erythraea* ER720(pASBVII).

D. Isolation, purification, and properties of 3- $\alpha$ -D-mycarosyl-5- $\beta$ -D-desosaminoyl-12-hydroxy-erythronolide B from *Sac. erythraea* ER720(pASBVII): *Sac. erythraea* ER720(pASBVII) is fermented for 3 days in SCM media with thiostrepton selection as described in General Methods. The fermentation broth is then cooled to 4°C and adjusted to pH 4.0 and extracted once with methylene chloride. The aqueous layer is readjusted to pH 9.0 and extracted twice with methylene chloride and the combined extracts are concentrated to a solid residue. This is digested in methanol and chromatographed over a column of Sephadex LH-20 in methanol. Fractions are tested for bioactivity against a sensitive organism, such as *Staphylococcus aureus* Th<sup>R</sup>, and active fractions are combined. The combined fractions are concentrated and the residue is digested in 10 ml of the upper phase of a solvent system consisting of n-heptane, benzene, acetone, isopropanol, 0.05 M, pH 7.0 aqueous phosphate buffer (5:10:3:2:5, v/v/v/v/v), and chromatographed on an Ito Coil Planet Centrifuge in the same system. Active fractions are combined, concentrated and partitioned between methylene chloride and dilute ammonium hydroxide (pH 9.0). The methylene chloride layer is separated and concentrated to yield the desired product as a white foam.

Example 3: Construction and characterization of *Streptomyces antibioticus* ATCC 11891(pXB6) that produces 3-des-oleandrosyl-3-mycarosyl oleandomycin

A. Construction of plasmid pKB6 and intermediates (see FIG. 9)

i) Construction of plasmid pK1: The DNA sequences of pBR322 (GenBank Accession #: J01749) and pUC19 (GenBank Accession #: X02514) are known. The 805 nt DNA segment comprising coordinates 1673 through 2478 of pBR322 is amplified by PCR employing two oligodeoxynucleotides, SEQ ID NO:35 (5'-GATCACATGTTCTTTCCCTG-CGTTATCCCCTG-3') and SEQ ID NO:36 (5'-GATCGGATCCATGCATGTCTAGAGCA-TCCGAGGATGCTGCTGGC-3'). After digestion of the purified PCR product with *A*/III and *Bam*HI the fragment is ligated into *A*/III and *Bam*HI digested pUC19 to give plasmid pK1. The identity of plasmid pK1, after transformation and isolation from *E. coli*, is verified by *Pvu*II digestion which releases fragments of 0.55 kb and 2.55 kb. Plasmid pK1 contains the ROP region of pBR322 that controls plasmid copy number.

ii) Construction of plasmid pKB1: The 2.24 kb DNA segment that carries the

*eryBIV* and *eryBV* genes, comprised between coordinates 56 and 2296 of the sequence presented in SEQ ID NO:2, is amplified by PCR employing two deoxyoligonucleotides, SEQ ID NO:37 (5'-GAATGCATCCTGGAAAGCGAGCAAATGCTCCGGTG-3') and SEQ ID NO:38 (5'-GATCTAGAGCTAGCCGGCGTGGCGGCGCGTG-3'). After digestion with  
5 *NsiI* and *XbaI* the fragment is ligated into *NsiI* and *XbaI* digested pK1 to yield plasmid pKB1, 5.3 kb in size. The identity of plasmid pKB1, after transformation and isolation from *E. coli*, is verified by *KpnI* digestion which releases fragments of 0.72 kb, 1.14 kb and 3.42 kb.

iii) Construction of plasmid pKB2: The 1.56 kb DNA segment that carries the *eryBVI* gene, comprised between coordinates 3121 and 4677 of the sequence presented in  
10 SEQ ID NO:2, is amplified by PCR employing two deoxyoligonucleotides, SEQ ID NO:39 (5'-GATCGCTAGCCGTGACCGGACCCTTACAGTGAGTG-3') and SEQ ID NO:40 (5'-GATCTAGACTTAAGTCATCCGGCGGTCCTGGTGTAGACGGC-3'). After digestion with *NheI* and *XbaI* the fragment is ligated into *NheI* and *XbaI* digested pKB1 to give plasmid pKB2, 6.9 kb in size. The identity of plasmid pKB2, after transformation and isolation from  
15 *E. coli*, is confirmed by *BamHI* digestion which releases fragments of 0.22 kb, 0.40 kb, 2.6 kb and 3.7 kb.

iv) Construction of plasmid pKB3: The 0.6 kb DNA segment that carries the *eryBVII* gene, comprised between coordinates 7385 and 7987 of the sequence presented in SEQ ID NO:2, is amplified by PCR employing two deoxyoligonucleotides, SEQ ID NO:41  
20 (5'-GATCTTAAGAACCGGAGTTGCGAGTACGTGAGCTGGCG-3') and SEQ ID NO:42 (5'-GATCTAGACCTAGGTCACCTGCCGGTGCTGGCGGGCTC-3'). After digestion with *AflIII* and *XbaI* the fragment is ligated into *AflIII* and *XbaI* digested pKB2 giving plasmid pKB3, 7.5 kb in size. The identity of plasmid pKB3, after transformation and isolation from *E. coli*, is verified by *PstI* digestion which releases fragments of 1.1 kb and 6.4 kb.

25 v) Construction of plasmid pKB4: The 1.0 kb DNA segment that carries the *eryBVII* gene, comprised between coordinates 2385 and 3410 of the sequence presented in SEQ ID NO:1, is amplified by PCR employing two deoxyoligonucleotides, SEQ ID NO:43 (5'-GATCCTAGGCCGCGAGGAAGGAGAGAACCACG-3') and SEQ ID NO:44 (5'-GATCTAGATTAATCACTGCAACCAGGCTTCCGGC-3'). Following digestion with  
30 *AvrII* and *XbaI* the fragment is ligated into *AvrII* and *XbaI* digested pKB3 yielding the desired plasmid pKB4. After transformation and isolation of the plasmid from *E. coli*, the identity of pKB4, 8.5 kb in size, is verified by *BglIII* and *EcoRI* digestion which releases fragments of 0.41 kb, 1.6 kb, 3.1 kb and 3.4 kb.

vi) Construction of plasmid pKB5: The DNA sequence of *eryBIII* has been  
35 reported (Haydock *et al* (1991) *Mol Gen Genet* 230:120). The 1.3 kb DNA segment that carries the *eryBIII* gene, comprised between coordinates 3965 and 5232 of the sequence depicted in Haydock *et al*, is amplified by PCR employing two deoxyoligonucleotides, SEQ

ID NO:45 (5'-GATTAATTGGCCGCGGCGCCGCTC-GTTATG-3') and SEQ ID NO:46 (5'-GATCTAGATAATTAATCATACGACTTCCAGTC-GGGGTAG-3'). After digestion with *MseI* and *XbaI* the fragment is ligated into *MseI* and *XbaI* digested pKB4 to give the desired plasmid pKB5, 9.8 kb in size. The identity of pKB5, after transformation and isolation from *E. coli*, is verified by *PstI* digestion which releases fragments of 1.1 kb, 2.5 kb, and 6.1 kb, visualized by gel electrophoresis.

vii) Construction of plasmid pKB6: The *eryBI* gene has been mapped (Haydock *et al* (1991) *Mol Gen Genet* 230:120) and the DNA sequence on both flanks of *eryBI* is known (Haydock *et al* (1991) *Mol Gen Genet* 230:120) and GenBank Accession # M11200. The 2.5 kb DNA segment that carries the *eryBI* gene, comprised between coordinates 1.1 and 3.6 of the map presented in Haydock *et al.*, is amplified by PCR employing two deoxyoligonucleotides: SEQ ID NO:47 (5'-GATTAATTAATGATCA-AGCTGAAAATTGTTTGCATG-3') and SEQ ID NO:48 (5'-GATCTAGACTGCCGGCT-CAGCCTTCCCAGGTTTCG-3'). After digestion with *PacI* and *XbaI* the fragment is ligated into *PacI* and *XbaI* digested pKB5 to give plasmid pKB6, 12.3 kb in size. The identity of pKB6, after transformation and isolation from *E. coli*, is verified by *BamHI* digestion which releases fragments of 0.22 kb, 0.40 kb, 1.4 kb, 2.6 kb, 3.3 kb and 4.4 kb. Plasmid pKB6 carries all of the *eryB* genes, *eryBI-eryBVII*, that are involved in the biosynthesis of mycarose and its attachment to the polyketide.

B. Construction of Plasmid pXSB6 (see FIG. 11): The 9.2 kb *NsiI-XbaI* segment of pKB6, prepared as described in Example 3(A)(vii) above, that carries all of the *eryB* genes is isolated and ligated into *PstI-XbaI* digested pASX2, prepared as described in Example 2(A) above, to give plasmid pXSB6. After transformation and isolation of the plasmid from *E. coli*, the identity of pXSB6, 17.2 kb in size, is verified by the observation of fragments of 0.41 kb, 1.9 kb, and 14.9 kb after *EcoRI* digestion. Plasmid pXSB6 carries all of the *eryB* genes in a transcriptional fusion downstream of the *ermE\** promoter on an *E. coli-Streptomyces* shuttle plasmid.

#### C. Construction of Plasmid pXB6

i) Construction of plasmid pN702 (see FIG. 10): Two oligonucleotides of the sequences: SEQ ID NO:49 5'-GGAATTCAGATCTATGCATTCTAGAA-3') and SEQ ID NO:50 (5'-CGCGTTCTAGAATGCATAGATCTGAATTCCTGCA-3') that include restriction enzyme sites for the enzymes *EcoRI*, *BglII*, *NsiI*, and *XbaI* and overhanging ends compatible with *PstI* and *MluI* are synthesized. Approximately 250 ng of each oligonucleotide are then mixed together in TE buffer and heated to 99°C for 1 min. After the solution is cooled slowly to room temperature allowing the oligonucleotides to anneal due to self complementarity, the annealed oligonucleotides are ligated into *PstI-MluI* digested pIJ702 to yield the desired plasmid pN702. After transformation and isolation of the plasmid

from *Streptomyces lividans* 1326, the identity of plasmid pN702, 4.3 kb in size, is verified by the observation of fragments of 0.75 kb and 3.6 kb after *EcoRI-BamHI* or *XbaI-BamHI* digestion.

5       ii) Construction of plasmid pX1 (see FIG. 10): The 290 bp *EcoRI-BamHI* segment that carries the *ermE*\* promoter is isolated from plasmid pIJ4070 and ligated into *EcoRI-BglIII* digested pN702 to give plasmid pX1. The resulting mixture contains the desired plasmid pX1. After transformation and isolation of the plasmid from *Streptomyces lividans* 1326, the identity of plasmid pX1, 4.6 kb in size, is verified by the observation of fragments of 1.0 kb and 3.6 kb after *NsiI-BamHI* digestion.

10       iii) Construction of plasmid pXB6 (see FIG. 11): The 9.2 kb *NsiI-XbaI* segment of pKB6, prepared as described in Example 3(A)(vii) above, that carries all of the *eryB* genes is isolated and ligated into *NsiI-XbaI* digested pX1 to give the desired plasmid pXB6. After transformation and isolation of the plasmid from *Streptomyces lividans* 1326, the identity of plasmid pXB6, 13.8 kb in size, is verified by the observation of fragments of  
15       0.41 kb, 1.9 kb, and 11.5 kb after *EcoRI* digestion. Plasmid pXB6 carries all of the *eryB* genes in a transcriptional fusion to the *ermE*\* promoter on a *Streptomyces* plasmid.

      D. Construction of *Streptomyces antibioticus* ATCC 11891(pXB6): Approximately 500 µg of plasmid pXB6, isolated from *Streptomyces lividans* 1326(pXB6), are electroporated into the oleandomycin producer *Streptomyces antibioticus* ATCC 11891 and  
20       several of the resulting Thio<sup>R</sup> colonies that appear on the R3M-agar plates containing thiostrepton are analyzed for their plasmid content. The presence of plasmid pXB6, 13.8 kb in size, is verified by the observation of fragments of 0.41 kb, 1.9 kb, and 11.5 kb after *EcoRI* digestion.

      E. Isolation, purification, and properties of 3-des-oleandrosyl-3-mycarosyl  
25       oleandomycin from *Streptomyces antibioticus* ATCC 11891(pXB6): *Streptomyces antibioticus* ATCC 11891(pXB6) is fermented for 5 days in SCM media with thiostrepton selection as described in General Methods. The fermentation broth is then cooled to 4°C and adjusted to pH 4.0 and extracted once with methylene chloride. The aqueous layer is readjusted to pH 9.0 and extracted twice with methylene chloride and the combined extracts  
30       are concentrated to a solid residue. This is digested in methanol and chromatographed over a column of Sephadex LH-20 in methanol. Fractions are tested for bioactivity against a sensitive organism, such as *Staphylococcus aureus* Th<sup>R</sup>, and active fractions are combined. The combined fractions are concentrated and the residue is digested in 10 ml of the upper phase of a solvent system consisting of n-heptane, benzene, acetone, isopropanol, 0.05 M, pH  
35       7.0 aqueous phosphate buffer (5:10:3:2:5, v/v/v/v/v), and chromatographed on an Ito Coil Planet Centrifuge in the same system. Closely eluting active fractions are combined, concentrated and partitioned between methylene chloride and dilute ammonium hydroxide

(pH 9.0). The methylene chloride layer is separated and concentrated to yield the desired product as a white foam.

Example 4: Construction and characterization of *Streptomyces violaceoniger* NRRL 2834(pXC4) that produces 5-des-chalcosyl-5-desosaminoyl lankamycin

A. Construction of plasmid pKC4 and intermediates (see FIG. 12)

i) Construction of plasmid pKC1: The 2.4 kb DNA segment that carries the *eryCII* and *eryCIII* genes, comprised between coordinates 33 and 2413 of the sequence presented in SEQ ID NO:1, is amplified by PCR employing two deoxyoligonucleotides, SEQ ID NO:51 (5'-GAATGCATCTGGCTGGGCGGAGGGAATTCATG-3') and SEQ ID NO:52 (5'-GATCTAGACTTAAGTCATCGTGGTTCTCTCCTTCCTGCGGC-3'). After digestion with *NsiI* and *XbaI* the purified PCR fragment is ligated into *NsiI* and *XbaI* digested pK1 to give plasmid pKC1, 5.5 kb in size. The identity of plasmid pKC1, after transformation and isolation from *E. coli*, is verified by *EcoRI* digestion which releases fragments of 2.2 kb and 3.3 kb.

ii) Construction of plasmid pKC2: The 732 bp DNA segment that carries the *eryCVI* gene, comprised between coordinates 2331 and 3063 of the sequence presented in SEQ ID NO:2, is amplified by PCR employing two deoxyoligonucleotides, SEQ ID NO:53 (5'-GATCCTTAAGCTCCGGAGGGAGCAGGGATG-3') and SEQ ID NO:54 (5'-GATCTAGACCTAGGTCATCCGCGCACACCGACGAAC-3'). After digestion with *AflIII* and *XbaI* the purified PCR fragment is ligated into *AflIII* and *XbaI* digested pK1 to give plasmid pKC2, 6.2 kb in size. The identity of plasmid pKC2, after transformation and isolation from *E. coli*, is verified by *XbaI-EcoRI* digestion which releases fragments of 0.95 kb, 2.2 kb and 3.1 kb.

iii) Construction of plasmid pKC3: The 2.7 kb DNA segment that carries the *eryCIV* and *eryCV* genes, comprised between coordinates 4650 and 7386 of the sequence presented in SEQ ID NO:2, is amplified by PCR employing two deoxyoligonucleotides, SEQ ID NO:55 (5'-GATCCTAGGCCGTCTACACCAGGACCGCCGG-3') and SEQ ID NO:56 (5'-GATCTAGATTAATCACCTTCCGCGCAGGAAGCCGC-3'). After digestion with *AvrII* and *XbaI* the purified PCR fragment is ligated into *AvrII* and *XbaI* digested pKC2 to yield plasmid pKC3, 9.0 kb in size. The identity of plasmid pKC3, after transformation and isolation from *E. coli*, is verified by *SphI* digestion which releases fragments of 4.0 kb and 5.0 kb.

iv) Construction of plasmid pKC4: The DNA sequence of the *eryCI* gene has been determined (GenBank Accession #X15541). The 1.1 kb DNA segment that carries the *eryCI* gene, comprised between coordinates 38 and 1161 of the sequence indicated above, is

amplified by PCR employing two deoxyoligonucleotides, SEQ ID NO:57 (5'-GATCTTAAG-CCGCCACTCGAACGGACACTCG-3') and SEQ ID NO:58 (5'-GATCTAGATCAAGCCC-CAGCCTTGAGGG-3'). After digestion with *MseI* and *XbaI* the fragment is ligated into *MseI* and *XbaI* digested pKC3 to give plasmid pKC4, 10.1 kb in size. The identity of plasmid pKC4, after transformation and isolation from *E. coli*, is verified by *KpnI* digestion which releases fragments of 0.15 kb, 0.31 kb, 4.1 kb and 5.5 kb. Plasmid pKC4 carries all of the *eryC* genes, *eryCI-eryCVI*, that are involved in the biosynthesis of desosamine and its attachment to the polydetide.

10 B. Construction of Plasmid pXSC4 (see FIG. 13): The 6.9 kb *NsiI-XbaI* segment of pKC4 that carries all of the *eryC* genes is isolated and ligated into *PstI-XbaI* digested pASX2, prepared as described in Example 2(A), to give the desired plasmid pXSC4, 14.9 kb in size, wherein all of the *eryC* genes are transcriptionally linked downstream of the *ermE\** promoter on an *E. coli-Streptomyces* shuttle plasmid. The identity of plasmid pXSC4, after transformation and isolation from *E. coli*, is verified by the observation of fragments of 0.29 kb, 2.2 kb, and 12.4 kb after *EcoRI* digestion.

15 C. Construction of Plasmid pXC4 (see FIG. 13): The 6.9 kb *NsiI-XbaI* segment of pKC4 that carries all of the *eryC* genes is isolated and ligated into *NsiI-XbaI* digested pX1, prepared as described in Example 3(C)(ii), to give the desired plasmid pXC4, 11.5 kb in size, wherein all of the *eryC* genes are transcriptionally linked downstream of the *ermE\** promoter on a *Streptomyces* plasmid. After transformation and isolation of the plasmid from *Streptomyces lividans* 1326, the identity of plasmid pXC4 is verified by the observation of fragments of 0.29 kb, 2.2 kb, and 9.0 kb after *EcoRI* digestion.

20 D. Construction of *Streptomyces violaceoniger* NRRL 2834(pXC4): Approximately 500 µg of the plasmid pXC4, isolated from *Streptomyces lividans* 1326(pXC4), are electroporated into the lankamycin producer *Streptomyces violaceoniger* NRRL 2834 and several of the resulting Thio<sup>R</sup> colonies that appear on the R3M-agar plates containing thiostrepton are analyzed for their plasmid content. The presence of plasmid pXC4 is verified by the observation of fragments of 0.29 kb, 2.2 kb, and 9.1 kb in size after *EcoRI* digestion of the plasmid.

30 E. Isolation, purification, and properties of 5-des-chalcosyl-5-desosaminoyl lankamycin: *S. violaceoniger* NRRL 2834(pXC4) is fermented for 5 days in SCM media with thiostrepton selection as described in General Methods. The fermentation broth is then cooled to 4°C and adjusted to pH 4.0 and extracted once with methylene chloride. The aqueous layer is readjusted to pH 9.0 and extracted twice with methylene chloride and the combined extracts are concentrated to a solid residue. This is digested in methanol and chromatographed over a column of Sephadex LH-20 in methanol. Fractions are tested for bioactivity against a sensitive organism, such as *Staphylococcus aureus* Th<sup>R</sup>, and active

fractions are combined. The combined fractions are concentrated and the residue is digested in 10 ml of the upper phase of a solvent system consisting of n-heptane, benzene, acetone, isopropanol, 0.05 M, pH 7.0 aqueous phosphate buffer (5:10:3:2:5, v/v/v/v/v), and chromatographed on an Ito Coil Planet Centrifuge in the same system. Active fractions are combined, concentrated and partitioned between methylene chloride and dilute ammonium hydroxide (pH 9.0). The methylene chloride layer is separated and concentrated to yield the desired product as a white foam.

Although the present invention is illustrated in the examples listed above in terms of preferred embodiments, these examples are not to be regarded as limiting the scope of the invention. The above illustrations serve to describe the principles and methodologies involved in creating the types of genetic alterations that can be introduced into *Sac. erythraea* and/or other *Streptomyces* that result in the synthesis of novel glycosylation-modified polyketide products. Although a single Type I alteration, leading to the production of for example, 4"-deoxy-4"-oxo-erythromycin A, is specified herein, it is obvious to those skilled in the art that other Type I changes can be introduced into the *eryB* and/or *eryC* genes leading to novel glycosylation-modified polyketide structures. Examples of additional Type I alterations leading to useful novel compounds include but are not limited to: mutations in the *eryBVII* gene conceivably leading to 3- $\alpha$ -D-mycarosyl-5- $\beta$ -D-desosaminoyl-12-hydroxy-erythronolide B and mutations in the *eryCVI* gene conceivably leading to N-3 $\alpha'$ -des-dimethyl erythromycin A. Moreover, it is obvious that Type I alterations in two or more different *eryB* and/or *eryC* genes can be combined leading to novel glycosylation-modified polyketide structures. Examples of combinations of two Type I alterations leading to useful compounds include but are not limited to: mutations in the *eryBIV* and *eryBVII* genes conceivably leading to 3- $\alpha$ -D-4"-deoxy-4"-oxo-mycarosyl-5- $\beta$ -D-desosaminoyl-12-hydroxy-erythronolide B; mutations in the *eryBIV* and *eryCVI* genes conceivably leading to 4"-deoxy-4"-oxo-(N-3 $\alpha'$ -des-dimethyl)-erythromycin A; and mutations in the *eryBIV*, *eryBVII*, and *eryCVI* genes conceivably leading to 3- $\alpha$ -D-4"-deoxy-4"-oxo-mycarosyl-5- $\beta$ -D-(N-3 $\alpha'$ -des-dimethyl)-desosaminoyl-12-hydroxy-erythronolide B. All Type I mutations or combinations of two or more Type I mutations in the *eryBII*, *eryBIV*, *eryBV*, *eryBVI*, *eryBVII*, *eryCII*, *eryCIII*, *eryCIV*, *eryCV*, or *eryCVI* genes, the *Sac. erythraea* strains that carry said mutations or combinations of mutations, and the corresponding polyketides produced from said strains, therefore, are included within the scope of the present invention.

Although the Type II mutation specified herein was constructed with the *eryBVII* gene on a self-replicating plasmid it is obvious that other *eryB* genes and *eryC* genes can be expressed in an antisense orientation leading to novel glycosylation-modified polyketide structures. Examples of additional Type II alterations leading to useful compounds include but are not limited to: antisense expression of the *eryBIV* gene conceivably leading to 4"-



deoxy-4"-oxo-erythromycin A and antisense expression of the *eryCVI* gene conceivably leading to N-3 $\alpha$ '-des-dimethyl erythromycin A. Moreover, it will occur to those skilled in the art that promoters other than the *ermE*\* promoter, for example the *melC* promoter of pIJ702, will be suitable for antisense expression, and that many self-replicating vectors in addition to pWHM4 will function to carry the antisense alteration. It will also occur to those skilled in the art that a self-replicating vector is not required for this invention and that the antisense alteration can be introduced directly into the chromosome using the same principles employed to construct a Type I gene alteration. An example of a Type II alteration that is introduced directly into the chromosome is the *eryBVII* antisense alteration described in Example 2 wherein DNA segments immediately upstream of the *eryK* gene are used to flank the *ermE-eryBVII*-phage fd terminator grouping in a pWHM3 vector, and this vector is integrated into and then resolved from the chromosome leaving the *ermE\*-eryBVII*-phage fd terminator grouping stably incorporated into this nonessential region of the chromosome of *Sac. erythraea* conceivably leading to the production of 3- $\alpha$ -D-mycarosyl-5- $\beta$ -D-desosaminoyl-12-hydroxy-erythronolide B. All Type II mutations in the *eryBII*, *eryBIV*, *eryBV*, *eryBVI*, *eryBVII*, *eryCII*, *eryCIII*, *eryCIV*, *eryCV*, or *eryCVI* genes whether carried on a self-replicating plasmid or integrated into a nonessential region of the chromosome, the *Sac. erythraea* strains that carry said mutations, and the corresponding polyketides produced from said strains, therefore, are included within the scope of the present invention.

Although Type III alterations, leading to the production of 5-des-chalcosyl-5-desosaminoyl lankamycin in *Streptomyces violaceoniger* and 3-des-oleandrosyl-3-mycarosyl oleandomycin in *Streptomyces antibioticus*, are specified herein, it is obvious that Type III alterations can be introduced into any polyketide producing microorganism leading to novel glycosylation modified polyketides. It will also occur to those skilled in the art that both the *eryB* and *eryC* genes can either be cotransformed into a polyketide producing microorganism or grouped together on a single vector that is introduced into a polyketide producing microorganism. An example of a Type III change using both the *eryB* and *eryC* genes together is their introduction into *Streptomyces violaceoniger* conceivably leading to 3-des-(4"-O-acetylarcansoyl)-3-mycarosyl-5-des-chalcosyl-5-desosaminoyl lankamycin. Although the Type III alterations specified herein have indicated a specific genetic order of the *eryB* or *eryC* genes, it will occur to those skilled at the art that many different genetic arrangements of the *eryB* or *eryC* genes will produce similar results. It will also that occur to those skilled at the art that certain arrangements of the *eryB* and/or *eryC* genes that lack one or more of the respective *eryB* and/or *eryC* genes will lead to the production of novel glycosylated polyketides in which intermediate compounds in the biosynthesis of mycarose and/or desosamine, respectively, such as those outlined in FIGS. 2 and 3, are attached to the polyketide. An example of a Type III alteration in which only a subset of the *eryB* and/or

*eryC* genes are used is the introduction of a pXC4 derivative that lacks the *eryCVI* gene, removed by digestion of plasmid pXC4 with *A*/III and *Avr*II followed by treatment with the Klenow fragment of DNA polymerase I and religation, into *Streptomyces violaceoniger* leading to the production of to 5-des-chalcosyl-5-(N-3 $\alpha$ '-des-dimethyl desosaminoyl) lankamycin. It will also that occur to those skilled at the art that promoters other than *ermE* or *ermE*\*, such as the *melC* promoter of plasmid pIJ702, and vectors other than pWHM4 or pIJ702 can also be utilized in the construction of a Type III alteration, and these variants are, of course, considered to be within the scope of the invention. Finally, it will also occur to those skilled in the art that a self-replicating vector is not required for this invention and that an assembly of sugar biosynthesis genes can be introduced directly into the chromosome of a heterologous host using the same principles employed to construct a Type I gene alteration once a nonessential region of the heterologous host chromosome has been identified. Alternatively, plasmids or bacteriophages which undergo site-specific recombination with host genes may also be used to introduce *eryB* and *eryC* genes into a host to effect Type III alterations. All Type III alterations using one or more of the *eryBII*, *eryBIV*, *eryBV*, *eryBVI*, *eryBVII*, *eryCII*, *eryCIII*, *eryCIV*, *eryCV*, or *eryCVI* genes, the polyketide producing strains that carry said alterations, and the corresponding polyketides produced from said strains, therefore, are included within the scope of the present invention.

In addition, it is also possible to create combinations of Type I and Type II alterations such that some Type I *eryB* and/or *eryC* mutations are introduced directly into the *Sac. erythraea* chromosome in the appropriate locus, while other *eryB* and/or *eryC* genes are inactivated by Type II alterations using a self-replicating or integrating vector. For example, combination of a Type I alteration, such as a mutation in *eryBIV*, and a Type II alteration, such as transformation with pASBVII, will conceivably lead to production of 3- $\alpha$ -D-4"-deoxy-4"-oxo-mycarosyl-5-B-D-desosaminoyl-12-hydroxy-erythronolide B. All combinations of two or more alterations of Type I and Type II, the *Sac. erythraea* strains that carry such alterations, and the glycosylated polyketides produced from such strains are included within the scope of the present invention.

As an extension of the examples reported with the *eryB* and/or *eryC* genes, it is possible to apply the method described herein to heterologous sugar biosynthesis genes that are similar to the *eryB* and/or *eryC* genes. The construction of strains carrying heterologous sugar biosynthesis genes that lead to the production of novel glycosylated polyketides requires: (i) cloning of the sugar biosynthesis genes from any other glycosylated-polyketide producing actinomycete, (ii) determining the nucleotide sequence of the cloned gene(s); (iii) excising and assembling the cloned gene(s) into vectors suitable for Type I, Type II, or Type III alterations; and (iv) transformation of polyketide producing microorganisms and screening for the novel compound. Any polyketide-associated sugar biosynthesis gene can thus be

precisely excised from the genome of a glycosylated polyketide producing microorganism and altered or arranged with other sugar biosynthesis genes and then introduced into the same or another polyketide producing microorganism to create a novel glycosylated polyketide of predicted structure. Thus, for example, a Type I or Type II alteration of a heterologous gene that is similar to an *eryB* and/or *eryC* gene, such as can be found in the *eryBVII* homolog for the synthesis of L-oleandrose in *Streptomyces antibioticus*, to result in the production of 3-des-L-oleandrosyl-3-D-oleandrosyl oleandomycin is included within the scope of the present invention. Similarly, a Type III assembly of the genes for the synthesis of a sugar other than mycarose or desosamine, such as can be found in the genes for the synthesis of angolosamine in *Streptomyces eurythermus*, and their transformation into *Sac. erythraea* to result in the synthesis of 5-des-desosaminoyl-5-angolosaminoyl-erythromycin A is included within the scope of the present invention.

It will occur to those skilled in the art that the Type I, Type II, and Type III genetic manipulations described herein and the polyketide producing microorganisms into which they are introduced are in no way exclusive. Hence, the choice of a convenient host and the choice of a Type I, Type II, or Type III alteration is based solely on the relatedness of the desired novel glycosylated polyketide to a natural counterpart. Therefore, Type I, Type II, and Type III alterations can be constructed in any polyketide producing microorganism employing either endogenous or exogenous sugar biosynthesis genes. Thus all Type I, Type II, and Type III mutations or various combinations thereof constructed in any polyketide producing microorganism according to the principles described herein, and the respective polyketides produced from such strains, are included within the scope of the present invention. Examples of glycosylated polyketides that can be altered by creating Type I, Type II, or Type III changes in the producing microorganisms include, but are not limited to macrolide antibiotics such as erythromycin, tylosin, spiramycin, etc; aromatic polyketides such as daunorubicin and doxorubicin, etc; polyenes such as candicidin, amphotericins, etc; and other complex polyketides such as avermectin.

Whereas the novel derivatives or modifications of erythromycin described herein have been specified as the A derivatives, such as 4"-deoxy-4"-oxo-erythromycin A, those skilled in the art understand that the wild type strain of *Sac. erythraea* produces a family of erythromycin compounds, including erythromycin A, erythromycin B, erythromycin C, and erythromycin D. Thus, modified strains of *Sac. erythraea*, such as strain ERBIV, for example, would be expected to produce the corresponding members of the 4"-deoxy-4"-oxo-erythromycin family, including 4"-deoxy-4"-oxo-erythromycin A, 4"-deoxy-4"-oxo-erythromycin B, 4"-deoxy-4"-oxo-erythromycin C, and 4"-deoxy-4"-oxo-erythromycin D. Similarly, all other modified strains of *Sac. erythraea* that produce novel glycosylated erythromycin derivatives would be expected to produce the A, B, C, and D forms of said

derivatives. For example, modified *Sac. erythraea* strains that produce 6-deoxyerythromycin, 6,12-dideoxyerythromycin and 6,7-anhydroerythromycin would be expected to produce novel glycosylation-modified polyketides by introduction of the additional modification of a Type I, II or III change in a sugar biosynthesis gene. Therefore, all members of the family of each of the novel erythromycins described herein or produced by these methods are included within the scope of the present invention.

Variations and modifications of the methods for obtaining the desired plasmids, hosts for cloning and choices of vectors and *eryB* and/or *eryC* genes to clone and modify, other than those described herein will occur to those skilled in the art. For example, although we have described the use of plasmids pWHM3, pWHM4, and pIJ702, other vectors can be employed wherein all or part of said plasmids is replaced by other DNA segments that function in a similar manner, such as replacing the pUC19 component of pWHM3 and pWHM4 with pBR322, available from BRL; or employing different segments of the pIJ101 replicon in pWHM3 and pIJ702, or the pJV1 replicon in pWHM4, respectively; or employing selectable markers other than thiostrepton- or ampicillin-resistance. These are just a few of a long list of possible examples all of which are included within the scope of the present invention. Similarly, the segments of the *eryB* and *eryC* loci that have been specified herein to generate the various Type I, Type II, and Type III alterations can readily be substituted for other segments of different length encoding the same functions, either produced by PCR-amplification of genomic DNA or of an isolated clone, or by isolating suitable restriction fragments from *Sac. erythraea*. In the same way it is possible to create Type I mutations functionally equivalent to those described herein by altering through deletion, insertion, or site directed mutagenesis different portions of the corresponding genes. It is also possible to create Type II mutations functionally equivalent to those described herein by employing larger or smaller portions of the corresponding genes; and it is possible to create Type III mutations using larger or smaller segments of the corresponding genes in the same or different linear order described herein. Additional modifications include changes in the restriction sites used for cloning or in the general methodologies described above. All such changes are included in the scope of the present invention. It will also occur to those skilled in the art that different methods are available to ferment *Sac. erythraea* and other polyketide producing microorganisms and to extract the novel polyketides specified herein, and all such methods are also included within the scope of this invention.

It will also be apparent that many modifications and variations of the invention as set forth herein are possible without departing from the spirit and scope thereof, and that, accordingly, such limitations are imposed only as indicated by the appended claims.

We claim:

1. An isolated single or double stranded polynucleotide having a nucleotide sequence which comprises (a) a nucleotide sequence selected from the group consisting of (i) the sense sequence of SEQ ID NO:1 from about nucleotide position 54 to about nucleotide position 1136; (ii) the sense sequence of SEQ ID NO:1 from about nucleotide position 1147  
5 to about nucleotide position 2412; (iii) sense sequence of SEQ ID NO:1 from about nucleotide position 2409 to about nucleotide position 3410; (iv) the sense sequence of SEQ ID NO:2 from about nucleotide position 80 to about nucleotide position 1048; (v) the sense sequence of SEQ ID NO:2 from about nucleotide position 1048 to about nucleotide position 2295; (vi) the sense sequence of SEQ ID NO:2 from about nucleotide position 2348 to about  
10 nucleotide position 3061; (vii) the sense sequence of SEQ ID NO:2 from about nucleotide position 3214 to about nucleotide position 4677; (viii) the sense sequence of SEQ ID NO:2 from about nucleotide position 4674 to about nucleotide position 5879; (iv) the sense sequence of SEQ ID NO:2 from about nucleotide position 5917 to about nucleotide position 7386; and (x) the sense sequence of SEQ ID NO:2 from about nucleotide position 7415 to  
15 about nucleotide position 7996;  
(b) sequences complementary to the sequences of (a);  
(c) sequences that, on expression, encode a polypeptide encoded by the sequences of (a); and  
(d) analogous sequences that hybridize under stringent conditions to the  
20 sequences of (a).
2. The polynucleotide of claim 1 that is a DNA molecule or RNA molecule.
3. The polynucleotide of claim 2 wherein the nucleotide sequence is the nucleotide sequence of (a) selected from the group consisting of (i) the sense sequence of SEQ ID NO:1 from about nucleotide position 54 to about nucleotide position 1136; (ii) the sense sequence of SEQ ID NO:1 from about nucleotide position 1147 to about nucleotide position 2412; (iii)  
5 the sense sequence of SEQ ID NO:2 from about nucleotide position 2348 to about nucleotide position 3061; (iv) the sense sequence of SEQ ID NO:2 from about nucleotide position 4674 to about nucleotide position 5879; and (v) the sense sequence of SEQ ID NO:2 from about nucleotide position 5917 to about nucleotide position 7386.
4. The polynucleotide of claim 2 wherein the nucleotide sequence is the nucleotide sequence of (a) selected from the group consisting of (i) sense sequence of SEQ ID NO:1 from about nucleotide position 2409 to about nucleotide position 3410; (ii) the sense

sequence of SEQ ID NO:2 from about nucleotide position 80 to about nucleotide position 1048; (iii) the sense sequence of SEQ ID NO:2 from about nucleotide position 1048 to about nucleotide position 2295; (iv) the sense sequence of SEQ ID NO:2 from about nucleotide position 3214 to about nucleotide position 4677; and (v) the sense sequence of SEQ ID NO:2 from about nucleotide position 7415 to about nucleotide position 7996.

5. The polynucleotide of claim 2 wherein the nucleotide sequence is the nucleotide sequence of (a) having the sense sequence of SEQ ID NO:2 from about nucleotide position 80 to about nucleotide position 1048.

6. A vector comprising the DNA molecule of claim 2.

7. The vector of claim 6 further comprising an enhancer-promoter operatively linked to the polynucleotide.

8. The vector of claim 6 wherein the polynucleotide has the nucleotide sequence of claim 5.

9. A host cell transformed with the vector of claim 6 or claim 7 or claim 8.

10. The transformed host cell of claim 9 that is a bacterial cell.

11. The transformed host cell of claim 10 wherein the bacterial cell is selected from the group consisting of *Streptomyces* and *E. coli*.

12. A method for directing the biosynthesis of specific glycosylation-modified polyketides by genetic manipulation of a polyketide-producing microorganism, said method comprising the steps of:

- 5 (1) isolating a sugar biosynthesis gene-containing DNA sequence according to claim 1;
- (2) identifying within said gene-containing DNA sequence one or more DNA fragments responsible for the biosynthesis of a polyketide-associated sugar or its attachment to a polyketide;
- (3) creating one or more specified changes into said DNA fragment or fragments, thereby resulting in an altered DNA sequence;
- 10 (4) introducing said altered DNA sequence into a polyketide-producing microorganism to replace the original sequence, said altered DNA sequence, when translated,

resulting in altered enzymatic activity capable of effecting the production of said specific glycosylation-modified polyketide;

- 15 (5) growing a culture of said altered polyketide-producing microorganism under conditions suitable for the formation of said specific glycosylation-modified polyketide; and  
(6) isolating said specific glycosylation-modified polyketide from said culture.

13. The method of claim 12 wherein said specified change in said DNA fragment or fragments results in the inactivation of at least one enzymatic activity involved in the biosynthesis of a polyketide-associated sugar or in its attachment to a polyketide.

14. The method of claim 13 wherein said polyketide-associated sugar is L-mycarose.

15. The method of claim 13 wherein said polyketide-associated sugar is D-desosamine.

16. A method for directing the biosynthesis of specific glycosylation-modified polyketides by genetic manipulation of a polyketide-producing microorganism, said method comprising the steps of:

- 5 (1) isolating a sugar biosynthesis gene-containing DNA sequence according to claim 1;  
(2) identifying within said gene-containing DNA sequence one or more DNA fragments responsible for the biosynthesis of a polyketide-associated sugar or its attachment to a polyketide;  
(3) reversing the strand orientation of said DNA fragment or fragments, thereby  
10 resulting in an altered DNA sequence which, when transcribed, results in production of an antisense mRNA;  
(4) introducing said altered DNA sequence into a polyketide-producing microorganism having an mRNA capable of binding to said antisense mRNA to produce an altered polyketide-producing microorganism capable of producing said specific  
15 glycosylation-modified polyketide;  
(5) growing a culture of said altered polyketide-producing microorganism under conditions suitable for the formation of said specific glycosylation-modified polyketide; and  
(6) isolating said specific glycosylation-modified polyketide from said culture.

17. A method for directing the biosynthesis of specific glycosylation-modified polyketides by genetic manipulation of a polyketide-producing microorganism, said method comprising the steps of:

- (1) isolating a sugar biosynthesis gene-containing DNA sequence according to claim

5 1;

(2) identifying within said gene-containing DNA sequence one or more DNA fragments responsible for the biosynthesis of a polyketide-associated sugar or its attachment to a polyketide;

(3) introducing said DNA fragment or fragments into a distinct polyketide-producing  
10 microorganism to produce an altered polyketide-producing microorganism capable of producing said specific glycosylation-modified polyketide;

(4) growing a culture of said polyketide-producing microorganism containing said DNA fragment or fragments under conditions suitable for the formation of said specific glycosylation-modified polyketide; and

15 (6) isolating said specific glycosylation-modified polyketide from said culture.

18. The method of claim 13 or claim 16 or claim 17 wherein said DNA fragment comprises one or more genes which encode an enzymatic activity involved in the biosynthesis of L-mycarose or in its attachment to a polyketide.

19. The method of claim 13 or claim 16 or claim 17 wherein said DNA fragment comprises one or more genes which encode an enzymatic activity involved in the biosynthesis of D-desosamine or in its attachment to a polyketide.

20. The method of claim 13 or claim 16 or claim 17 wherein said DNA fragment is the sequence of claim 8.

21. An isolated polypeptide having an amino acid sequence encoded by a nucleotide sequence selected from the group consisting of the sense sequence of SEQ ID NO:1 from about nucleotide position 54 to about nucleotide position 1136; the sense sequence of SEQ ID NO:1 from about nucleotide position 1147 to about nucleotide position 2412; sense sequence  
5 of SEQ ID NO:1 from about nucleotide position 2409 to about nucleotide position 3410; the sense sequence of SEQ ID NO:2 from about nucleotide position 80 to about nucleotide position 1048; the sense sequence of SEQ ID NO:2 from about nucleotide position 1048 to about nucleotide position 2295; the sense sequence of SEQ ID NO:2 from about nucleotide position 2348 to about nucleotide position 3061; the sense sequence of SEQ ID NO:2 from  
10 about nucleotide position 3214 to about nucleotide position 4677 ; the sense sequence of SEQ ID NO:2 from about nucleotide position 4674 to about nucleotide position 5879; the sense sequence of SEQ ID NO:2 from about nucleotide position 5917 to about nucleotide position 7386; and the sense sequence of SEQ ID NO:2 from about nucleotide position 7415 to about nucleotide position 7996.



22. An isolated polypeptide of claim 31 encoded by the sequence of SEQ ID NO:2 from about nucleotide position 80 to about nucleotide position 1048.

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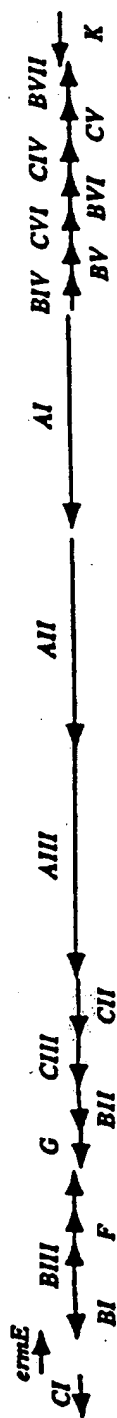


FIG. 1A

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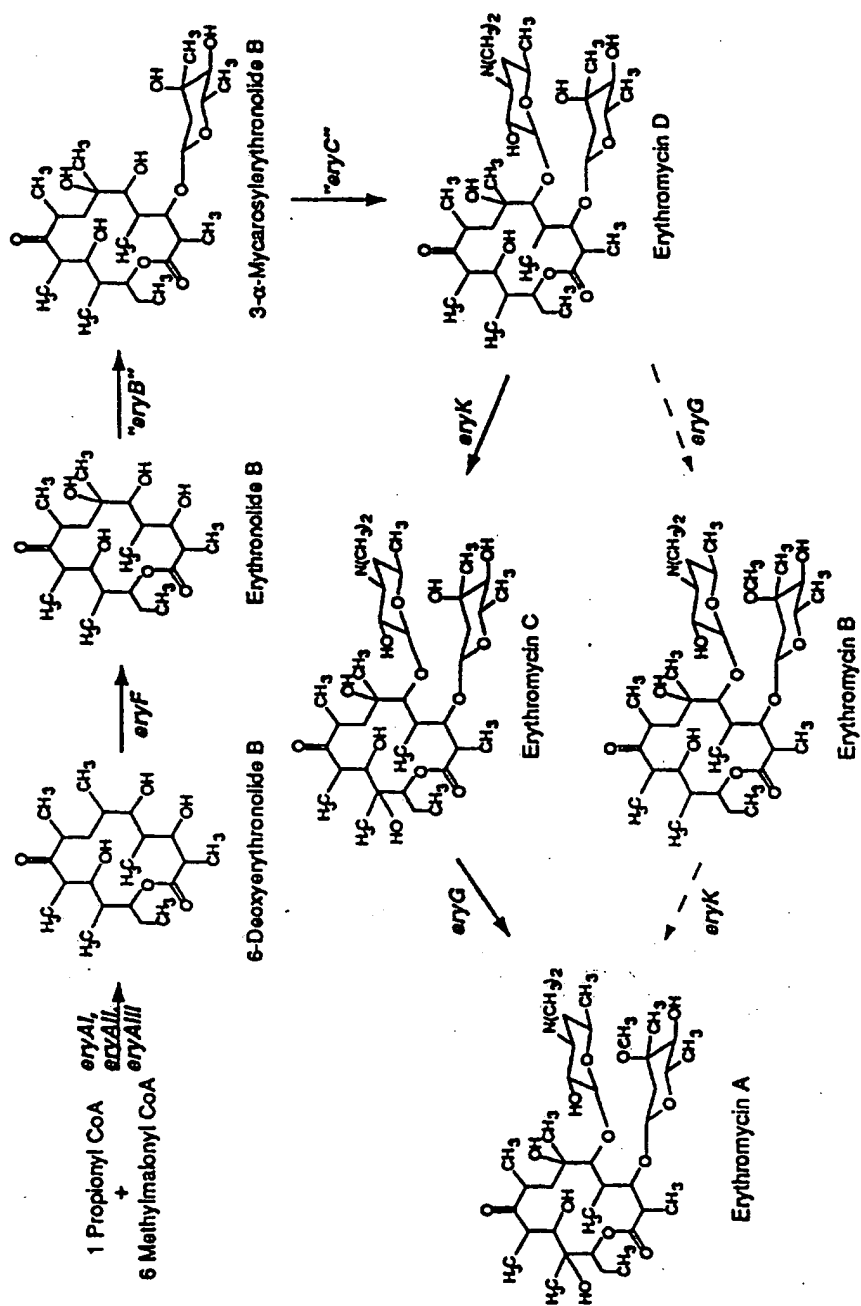


FIG. 1B

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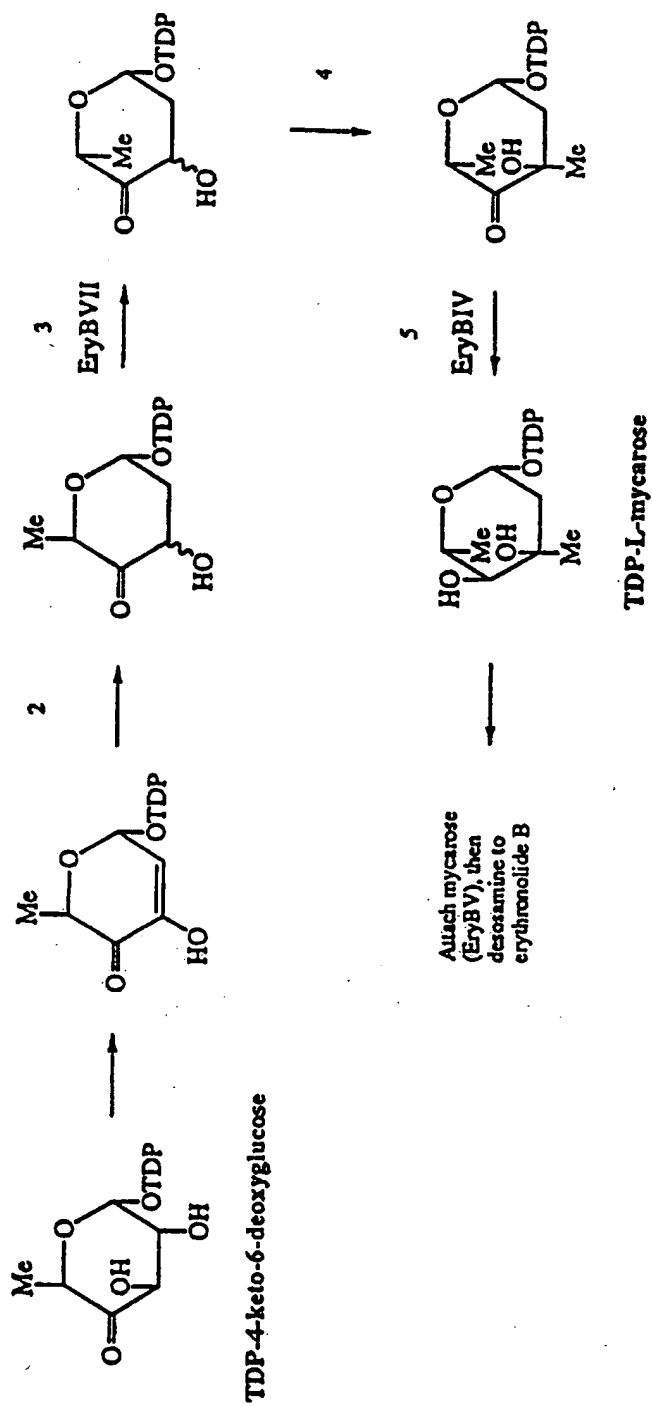


FIG. 2

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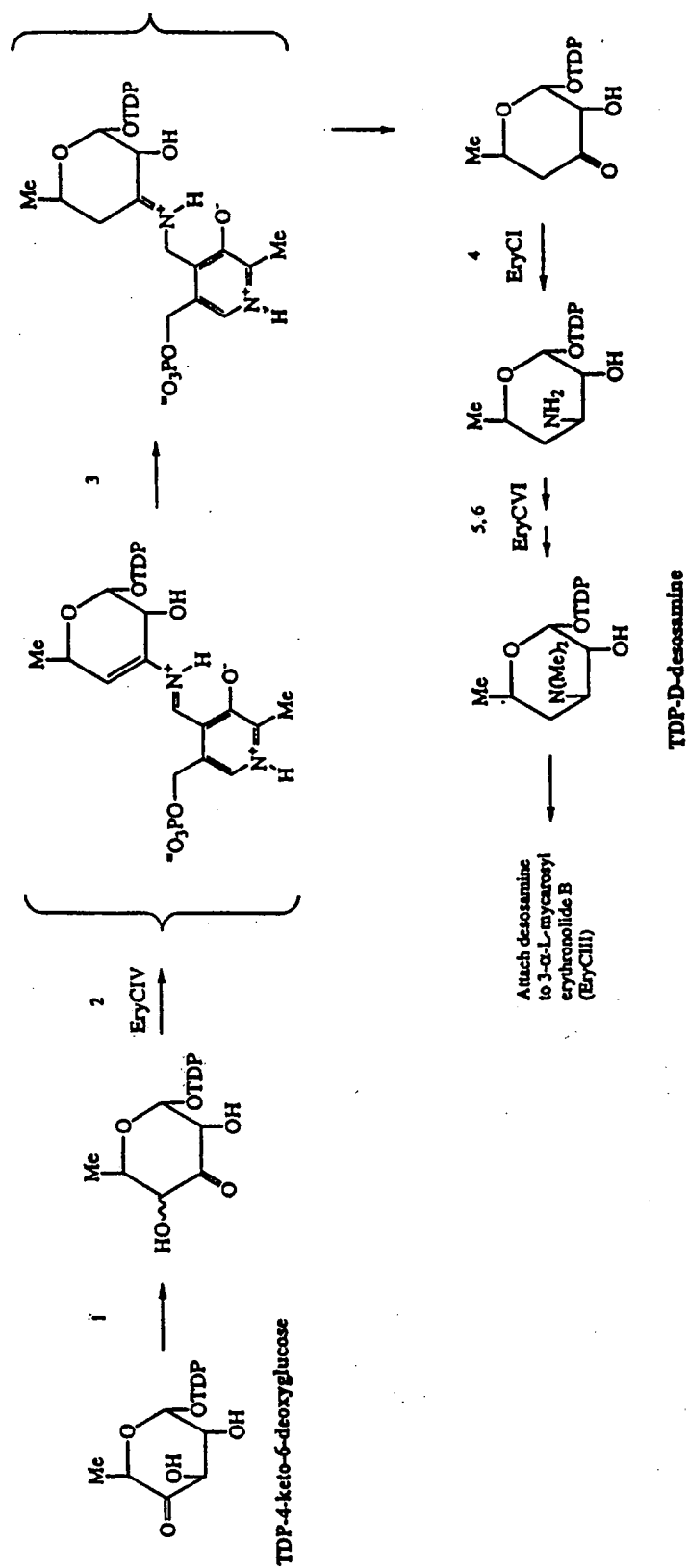


FIG. 3

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1 CACGCCGACGCGATCGCGCGGCACATCGACGCTGGCTGGCGGAGGGAATTCATGACCA 60  
M T T

61 CGACCGATCGCGCGGGCTGGGCAGGCAGCTCCAGATGATCCGCGCCTGCACTGGGGTT 120  
T D R A G L G R Q L Q M I R G L H W G Y

121 ACGGCAGCAACGGCGACCCTTACCCGATGCTGCTGTGCGGACACGACGACCCCGCAGC 180  
G S N G D P Y P M L L C G H D D D P Q R

181 GCCGGTACCGCTCGATGCGCGAGTCCGGTGTGCGGCGCAGGACCGAGACGTGGGTGGTGG 240  
R Y R S M R E S G V R R R T E T W V V A

241 CCGACCACGCCACCGCCCGGCAGGTGCTCGACGACCCCGCTTACCCGCGCCACCGGAC 300  
D H A T A R Q V L D D P A F T R A T G R

301 GCACACCGGAATGGATGCGGGCCGCGGGCGGCCACCCGCGAGTGGGCCCAGCCGTTCC 360  
T P E W M R A A G A P P A E W A Q P F R

361 GGGACGTGCACGCCGCTCCTGGGAAGGCCAGGTCCCCGACGTCGGGGAACCTGGCGGAGA 420  
D V H A A S W E G E V P D V G E L A E S

421 GCTTCGCCGGTCTGCTCCCCGGCGCGGGCGCGCGGCTGGACCTGGTCGGCGGACTTCGCCT 480  
F A G L L P G A G A R L D L V G D F A W

481 GGCAGGTACCGGTGCAGGGCATGACCGCCGTGCTCGGCGCAGCCGGAGTGCTGCGCGGGC 540  
Q V P V Q G M T A V L G A A G V L R G A

541 CCGCGTGGGACGCCCGCTCAGCCTGGACGCCAGCTCAGCCCGCAGCAGCTCGCGGTGA 600  
A W D A R V S L D A Q L S P Q Q L A V T

601 CCGAAGCAGCGGTGCGGGCACTGCCCGCCGACCCGCACTGCGCGCCCTGTTGCGCGGGG 660  
E A A V A A L P A D P A L R A L F A G A

661 CCGAGATGACCGCGAACACCGTGGTCGACGCGGTCTGGCCGTCTCGGCGGAACCGGGC 720  
E M T A N T V V D A V L A V S A E P G L

721 TGGCCGAACGGATCGCCGACGACCCCGCCCGCGCAGCGAACCCTCGCCGAGGTGCTGC 780  
A E R I A D D P A A A Q R T V A E V L R

781 GCCTGCACCCGGCATTGCACCTGGAGCGGGCGCACGGCCACCGCAGAGGTGCGGCTCGGCG 840  
L H P A L H L E R R T A T A E V R L G E

841 AGCACGTGATCGGCGAAGGCGAGGAGTCTGCTGCTGCTGCGGCGGGCCAAACCGCGACC 900  
H V I G E G E E V V V V V A A A N R D P

901 CCGAGGTCTTCGCGGAGCCCGACCGCCTCGACGTGGACCCCGGACCGCGGACCGCGCGC 960  
E V F A E P D R L D V D R P D A D R A L

FIG. 4A-1

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961 TGTCGGCACATCGCGGCCACCCCGGCAGGCTGGAGGAGCTGGTCACCGCGCTCGCCACCG 1020  
S A H R G H P G R L E E L V T A L A T A

1021 CCGCACTGCGGGCCGCGGCCAAGGCGCTGCCCGGACTCAGCCCCAGCGGCCCGGTCTGTC 1080  
A L R A A A K A L P G L T P S G P V V R

1081 GCGCGCGCGATCACCCGTCCTGCGGGGAACCAACCGCTGCCCGTCGAGCTCTGAGGAT 1140  
R R R S P V L R G T N R C P V E L \*

1141 TCCGCGATGCGCGTCTCTCTCTCCATGGCCAGCAAGGCCACCTCTTCGGCCTCGTC 1200  
M R V V F S S M A S K S H L F G L V

1201 CCCCTCGCATGGGCGTTCGCGCGCGGGGCACGAGGTCCGCGTGGTCGCGTCCCCGGCG 1260  
P L A W A F R A A G H E V R V V A S P A

1261 CTCACCGAGGACATCACCGCGCGGGCTGACCGCGCTCCCGGTGGGCACCGACGTCGAC 1320  
L T E D I T A A G L T A V P V G T D V D

1321 CTCGTGGACTTCATGACCCACGCGGGCCACGACATCATCGACTACGTCCGGAGCCTGGAC 1380  
L V D F M T H A G H D I I D Y V R S L D

1381 TTCAGCGAGCGGGACCCCGCCACCTTGACCTGGGAGCACCTGCGGGGCATGCAGACCGTG 1440  
F S E R D P A T L T W E H L R G M Q T V

1441 CTCACCCCGACCTTCTACGCCCTGATGAGCCCGGACACGCTCATCGAAGGCATGGTCTCG 1500  
L T P T F Y A L M S P D T L I E G M V S

1501 TTCTGCCGGAAGTGGCGGCCGACCTGGTCATCTGGGAGCCGCTCACCTTCGCCGCGCCC 1560  
F C R K W R P D L V I W E P L T F A A P

1561 ATCGCGGGCGCGGTGACCGGAACGCGGCACGCGGGCTGCTGTGGGGACCCGACATCACC 1620  
I A G A V T G T P H A R L L W G P D I T

1621 ACCCGGGCGCGGCAGAACTTCTCGGCCTGCTGCCCGACAGCCGGAGGAGCACCGGGAG 1680  
T R A R Q N F L G L L P D Q P E E H R E

1681 GCGCCGCTCGCCGAGTGGCTCACCTGGACGCTGGAGAAGTACGCGGCCCGGCCTTCGAC 1740  
G P L A E W L T W T L E K Y G G P A F D

1741 GAGGAGGTGGTCGTGCGGGCAGTGGACGATCGACCCCGCCCGCGCGATCAGGCTCGAC 1800  
E E V V V G Q W T I D P A P A A I R L D

1801 ACCGGCCTGAAGACCGTCGGGATGCGCTACGTCGACTACAACGGGCCGTCCGTGGTGCCG 1860  
T G L K T V G M R Y V D Y N G P S V V P

1861 GAATGGCTGCACGACGAGCCCGAGCGCCGCGCGTGTGCCTCACGCTCGGGATCTCCAGC 1920  
E W L H D E P E R R R V C L T L G I S S

FIG. 4A-2

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1921 CGCGAGAACAGCATCGGGCAGGTCTCCATCGAGGAGCTGCTGGGTGCCGTCCGGCGACGTC 1980  
R E N S I G Q V S I E E L L G A V G D V

1981 GACGCCGAGATCATCGCGACCTTCGACGCGCAGCAGCTAGAAGGCGTCGCGAACATCCCG 2040  
D A E I I A T F D A Q Q L E G V A N I P

2041 CACAACGTCCGCACGGTCGGCTTCGTCCCGATGCACGCGCTGCTGCCGACCTGCCGCGCG 2100  
H N V R T V G F V P M H A L L P T C A A

2101 ACGGTGCACCACGCGCGACCCGGGAGCTGGCACACCGCGCGATCCACGGCGTGCCGCGAG 2160  
T V H H G G P G S W H T A A I H G V P Q

2161 GTGATCCTGCCCGACGGCTGGGACACCGGCGTGCGCGCGCAGCGCACGCGAGGAATTCGGG 2220  
V I L P D G W D T G V R A Q R T Q E F G

2221 GCGGGGATCGCGCTGCCCGTGCCCGAGCTGACCCCGACCGAGCTCCGGGAGTCGGTGAAG 2280  
A G I A L P V P E L T P D Q L R E S V K

2281 CGGGTCCTCGACGACCCGGGCCACCGCGCGCGCGCGGATGCGCGACGACATGCTC 2340  
R V L D D P A H R A G A A R M R D D M L

2341 GCGGAGCCGTACCGGCGGAGGTGCTCGGCATCTGCGAGGAAGTGGCCGAGGAAGGAGA 2400  
A E P S P A E V V G I C E E L A A G R R

2401 GAACCACGATGACCACCGACGCCGCGACGACGTCGCGCTCGGGCGTTCCGCGCTGCTCA 2460  
E P R \*  
M T T D A A T H V R L G R S A L L T

2461 CCAGCAGGCTCTGGCTCGGCACGGTGAACCTCAGCGGACCGCTCGAGGACGACGACGCGC 2520  
S R L W L G T V N F S G R V E D D D A L

2521 TGCGCCTGATGGACCACGCCCGGGACCGCGCATCAACTGCCTCGACACCGCGACATGT 2580  
R L M D H A R D R G I N C L D T A D M Y

2581 ACGGCTGGCGGCTCTACAAGGGCCACACCGAGGAGCTGGTGGGCAGGTGGCTGGCCGAGG 2640  
G W R L Y K G H T E E L V G R W L A Q G

2641 GCGGCGGACGGCGCGAGGACACCGTGTGCGGACCAAGGTGCGGCGGAGATGAGCGAGC 2700  
G G R R E D T V L A T K V G G E M S E R

2701 GCGTCAACGACAGCGGGCTGTGCGCGCGGCACATCATCGCCTCCTGCGAGGGATCGCTGC 2760  
V N D S G L S A R H I I A S C E G S L R

2761 GCAGGCTGGGCGTCGACCACATCGACGTCTACCAGATGCACCACATCGACCGGTCCGCGC 2820  
R L G V D H I D V Y Q M H H I D R S A P

2821 CGTGGGACGAGGTGTGGCAGGCCATGGACAGCCTCGTCGCCAGCGGCAAGGTCTCCTACG 2880  
W D E V W Q A M D S L V A S G K V S Y V

FIG. 4A-3



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2881 TCGGCTCGTGAACCTCGCGGGCTGGCACATCGCCGCCGCGCAGGAGAACGCCGCCGCC 2940  
G S S N F A G W H I A A A Q E N A A R R

2941 GCCACTCCCTGGGCATGGTCTCCACCACTGCCTGTACAACCTGGCGGTCCGGCAGCCG 3000  
H S L G M V S H Q C L Y N L A V R H A E

3001 AGCTGGAGGTGCTGCCCCGCCGCGAGGCCTACGGGCTCGGCGTCTTCGCTGGTCGCCG 3060  
L E V L P A A Q A Y G L G V F A W S P L

3061 TGCACGGCGGCTGCTCAGCGGAGCGCTGGAGAAGCTGGCCGCGGGCACC CGGTGAAGT 3120  
H G G L L S G A L E K L A A G T A V K S

3121 CGGCGCAGGGCCGTCGCGAGGTGCTGTTGCCGTCCTGCGCCCGCGGATCGAGGCCTACG 3180  
A Q G R A Q V L L P S L R P A I E A Y E

3181 AGAAGTTCTGCCGCAACCTCGGCGAAGACCCGCGGAGGTGGGGCTCGCATGGGTGCTGT 3240  
K F C R N L G E D P A E V G L A W V L S

3241 CCCGGCCCGGCATCGCCGGCGCGTCATCGGCCCGGAACCCCGAGCAGCTCGACTCCG 3300  
R P G I A G A V I G P R T P E Q L D S A

3301 CGCTGAAGGCGTCCGCGATGACCCTGGACGAGCAGGCGCTGTCCGAACCTGGACGAGATCT 3360  
L K A S A M T L D E Q A L S E L D E I F

3361 TCCCCGCGGTGGCCTCCGGCGGCGCGCGCGGAAGCCTGGTTGCAGTGAGCACAAGAGG 3420  
P A V A S G G A A P E A W L Q \*

3421 AACCGAGAAAGGATACGGCTGGTGAGCGTGAAGCAGAAGTCAGCGTTGCAGGACCTGGTC 3480

3481 GACTTCGCCAAGTGGCACGTGTGGACCAGGGTGCGGCCGTCCAGCCGTGCGGCCCTGGCC 3540

3541 TACGAGCTGTTCGCCGACGACCACGAGGCCACGACCGAGGGCGCCTACATCAACCTCGGC 3600

3601 TACTGGAAGCCCGGTGCGCCGGCCTGGAGGAGGCCAACAGGAGCTGGCGAACCAAGCTC 3660

3661 GCCGAGGCCGCGGGGATCAGCGAGGGCGACGAGGTGCTCGACGTCGGGTTCGGGCTCGGC 3720

3721 GCGCAGGACTTCTTCTGGCTCGACCTGCAGCCAGCT 3756

FIG. 4A-4

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1 CGGGTTGCCGCACATCGCGCTGGGGAGATTCTTTGAATTCGCCCCTAGCACCGACCTGG 60

61 AAAGCGAGCAAATGCTCCGGTGAATGGGATCAGTGATTCCCCCGTCAATTGATCACCCCT 120  
V N G I S D S P R Q L I T L

121 TCTGGGCGCTTCCGGCTTCGTGGGAGCGCGTTCGCGGAGCTGCGCGACCAACCCGGT 180  
L G A S G F V G S A V L R E L R D H P V

181 CCGGCTGCGCGCGGTGTCCCGCGGCGGAGCGCCCGCGGTTCGCGCGGCGCGCGGAGGT 240  
R L R A V S R G G A P A V P P G A A E V

241 CGAGGACCTGCGCGCCGACCTGCTGGAACCGGGCCGGCCCGCGCGATCGAGGACGC 300  
E D L R A D L L E P G R A A A A I E D A

301 CGACGTGATCGTGCACCTGGTGGCGCACGCAGCGGGCGGTTCACCTGGCGCAGCGCCAC 360  
D V I V H L V A H A A G G S T W R S A T

361 CTCCGACCCGGAAGCCGAGCGGGTCAACGTTCGGCCTGATGCACGACCTCGTCGGCGCGCT 420  
S D P E A E R V N V G L M H D L V G A L

421 GCACGATCGCCGCGAGGTGACGCGCCCGCTGTGCTCTACGCGAGCACCGCACAGGCCGC 480  
H D R R R S T P P V L L Y A S T A Q A A

481 GAACCCGTTCGGCGGCCAGCAGGTACGCGCAGCAGAAGACCGAGGCCGAGCGCATCCTGCG 540  
N P S A A S R Y A Q Q K T E A E R I L R

541 CAAAGCCACCGACGAGGGCCGGGTGCGCGGCGTGATCCTGCGGCTGCCCGCGGTCTACGG 600  
K A T D E G R V R G V I L R L P A V Y G

601 CCAGAGCGGCCCGTCCGGCCCCATGGGGCGGGCGTGGTCGCGAGCGATGATCCGGCGTGC 660  
Q S G P S G P M G R G V V A A M I R R A

661 CCTCGCCGGCGAGCCGCTCACCATGTGGCAGCAGCGCGCGTGGCGCGGACCTGCTGCA 720  
L A G E P L T M W B D G G V R R D L L H

721 CGTCGAGGACGTGGCCACCGCGTTCGCGCGCGCGCTGGAGCACCACGACGCGCTGGCCGG 780  
V E D V A T A F A A A L E H H D A L A G

781 CGGCACGTGGGCGCTGGGCGCCGACCGATCCGAGCCGCTCGGCGACATCTTCGGGGCGGT 840  
G T W A L G A D R S E P L G D I F R A V

841 CTCCGGCAGCGTCGCCCGGCGAGCCGGCAGCCCCCGCGTGCAGCTGGTCACCGTGCCCGC 900  
S G S V A R Q T G S P A V D V V T V P A

901 GCCCCAGCACGCCGAGGCCAACGACTTCCGCGAGCAGACATCGACTCCACCGAGTTCGG 960  
P E H A E A N D F R S D D I D S T E F R

FIG. 4B-1

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961 CAGCCGGACCGGCTGGCGCCCCCGGGTTTCCCTACCGACGGCATCGACCGGACGGTGGC 1020  
S R T G W R P R V S L T D G I D R T V A

1021 CGCCCTGACCCCCACCGAGGAGCACTAGTGGGGTACTGCTGACGTCCTTCGCGCACCGC 1080  
A L T P T E E H \*  
V R V L L T S F A H R

1081 ACGCACTTCCAGGGACTGGTCCCGTGGCGTGGGCGCTGCGCACCGGGTCACGACGTG 1140  
T H F Q G L V P L A W A L R T A G H D V

1141 CGCGTGGCGCCCCAGCCCGGCTCACCGACGGGTATCGGCGCCGGTCTCACCGCGGT 1200  
R V A A Q P A L T D A V I G A G L T A V

1201 CCCGTCGGCTCCGACCACCGGCTGTTGACATCGTCCCGAAGTCGCGCTCAGGTGCAC 1260  
P V G S D H R L F D I V P E V A A Q V H

1261 CGCTACTCCTTCTACCTGGACTTCTACCACCGGAGCAGGAGCTGCACTCGTGGGAGTTC 1320  
R Y S F Y L D F Y H R E Q E L H S W E F

1321 CTGCTCGGCATGCAGGAGGCCACCTCGCGGTGGGTATACCCGGTGGTCAACAACGACTCC 1380  
L L G M Q E A T S R W V Y P V V N N D S

1381 TTCGTCGCCGAGCTGGTCGACTTCGCCCGGACTGGCGTCTGACCTGGTGTCTGGGAG 1440  
F V A E L V D F A R D W R P D L V L W E

1441 CCGTTCACCTTCGCCCGCGCGCTCGCGGCCCGGGCTGCGGAGCCGCGCACGCCCGGCTG 1500  
P F T F A G A V A A R A C G A A H A R L

1501 CTGTGGGGCAGCGACCTACCGGCTACTTCCGCGCCGGTTCAGGCGCAACGCCTGCCA 1560  
L W G S D L T G Y F R G R F Q A Q R L R

1561 CGGCCGCCGGAGGACCGGCCGGACCCGCTGGGCACGTGGCTGACCGAGGTTCGCGGGGCGC 1620  
R P P E D R P D P L G T W L T E V A G R

1621 TTCGGCGTCGAATTCGGCGAGGACCTCGCGGTTCGGGCAGTGGTTCGGTCGACCAATTGCCG 1680  
F G V E F G E D L A V G Q W S V D Q L P

1681 CCGAGTTTCCGGCTGGACACCGGAATGGAACCGTTGTCCGCGGACCCCTGCCCTACAAC 1740  
P S F R L D T G M E T V V A R T L P Y N

1741 GCGCGCTCGGTGGTTCCGGACTGGCTCAAGAAGGGCAGTGCAGCTCGACGCATCTGCATT 1800  
G A S V V P D W L K K G S A T R R I C I

1801 ACCGGAGGGTTCTCCGGACTCGGGCTCGCCGCCGATGCCGATCAGTTCGCGCGGACGCTC 1860  
T G G F S G L G L A A D A D Q F A R T L

FIG. 4B-2

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1861 GCGCAGCTCGCGCGATTTCGATGGCGAAATCGTGGTTACGGGTTCCGGTCCGGATACTCC 1920  
A Q L A R F D G E I V V T G S G P D T S

1921 GCGGTACCGGACAAACATTCTGTTTGGTGGATTTCGTTCCGATGGGCGTTCTGCTCCAGAAC 1980  
A V P D N I R L V D F V P M G V L L Q N

1981 TGCGCGCGCATCATCCACCACGGCGGGCGGGAACCTGGGCCACGGCACTGCACCACGGA 2040  
C A A I I H H G G A G T W A T A L H H G

2041 ATTCCGCAAATATCAGTTGCACATGAATGGGATTGCATGCTACCGGGCCAGCAGACCGCG 2100  
I P Q I S V A H E W D C M L R G Q Q T A

2101 GAACTGGGCGCGGGAATCTACCTCCGGCCGACGAGGTGATGCCGACTCATTGGCGAGC 2160  
E L G A G I Y L R P D E V D A D S L A S

2161 GCCCTCACCCAGGTGGTCGAGGACCCACCTACACCGAGAACCGGTTGAAGCTTCGCGAG 2220  
A L T Q V V E D P T Y T E N A V K L R E

2221 GAGGCGCTGTCCGACCCGACGCCGAGGATCGTCCCGGACTGGAGGAACTCACGCGC 2280  
E A L S D P T P Q E I V P R L E E L T R

2281 CGCCACGCCGGCTAGCGGTTTCCGACCGACAAGTCCGTCGACAGCACACCTCCGGAGGG 2340  
R H A G \*

2341 AGCAGGGATGTACGAGGGCGGGTTCGCCGAGCTTACGACCGGTTCTACCGCGCCGGGG 2400  
M Y E G G F A E L Y D R F Y R G R G

2401 CAAGGACTACCGGCCGAGGCCGCGCAGGTGCGCGGGCTGGTCAGAGACCGCTGCCCTC 2460  
K D Y A A E A A Q V A R L V R D R L P S

2461 GGCTTCCTCGCTGCTCGACGTGGCCTGCCGGACCGGCACCCACCTGCGCCGGTTCCCGA 2520  
A S S L L D V A C G T G T H L R R F A D

2521 CCTCTCGACGACGTGACCGGGCTGGAGCTGTGCGCGGCGATGATCGAGGTCGCCCGGC 2580  
L F D D V T G L E L S A A M I E V A R P

2581 GCAGCTCGGCGGCATCCCGGTGCTGCAGGGCGACATGCGCGACTTCGCGCTGGATCGCGA 2640  
Q L G G I P V L Q G D M R D F A L D R E

2641 GTTCGACGCCGTCACTGCATGTTTCAGCTCCATCGGGCACATGCGCGACGGCGCCGAGCT 2700  
F D A V T C M F S S I G H M R D G A E L

2701 GGACCAGGCGCTGGCGTCTTCGCCCGCCACCTCGCCCCGGCGGCGTCTGTTGGTGGTCGA 2760  
D Q A L A S F A R H L A P G G V V V V E

2761 ACCGTGGTGGTTCCCGGAGGACTTCCTCGACGGCTACGTGGCGGTGACGTGGTGGCGGA 2820  
P W W F P E D F L D G Y V A G D V V R D

FIG. 4B-3

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2821 CGGCGACCTGACGATCTCGCGCTCTCGCACTCCGTGCGCGCCGGCGGCGGCGACCCGGAT 2880  
G D L T I S R V S H S V R A G G A T R M

2881 GGAGATCCACTGGGTCGTGGCCGACGCGGTGAACGGTCCGCGGCACCACGTGGAGCACTA 2940  
E I H W V V A D A V N G P R H H V E H Y

2941 CGAGATCAGCTCTTCGAGCGGCAGCAGTACGAGAAGGCCTTCACCGCGGCCGGTTGCGC 3000  
E I T L F E R Q Q Y E K A F T A A G C A

3001 TGTGCACTCCTGGAGGGCGGACCCCTCCGGACGCGGGTTGTTTCGTGGTGTGCGCGGATG 3060  
V Q Y L E G G P S G R G L F V G V R G \*

3061 ACCCGTGCCTCGCGTTTTCCGTTCCTGGCAGGTGATCCGCTCCACGGGCCCTTTCCCC 3120

3121 GCCGTGACCGGACCCTTACAGTGAGTGCGGGTCTTGATCGACAACGCCCGCGGCAGCAA 3180

3181 GCGGAGCCGTCGACGACACCGCAGGGAGAGTCGATGGGTGATCGGACCGGCGACCGGACG 3240  
M G D R T G D R T

3241 ATTCCGGAATCCTCGCAGACCGCAACCGCTTCTGCTCGCGGACGGCGGAATCCCCACC 3300  
I P E S S Q T A T R F L L G D G G I P T

3301 GCCACGGCGGAACCCACGACTGGCTGACCCGCAACGGCGCCGAGCAGCGGCTCGAGGTG 3360  
A T A E T H D W L T R N G A E Q R L E V

3361 GCGCGCGTGCCGTTTCAGCGCCATGGACCGTGGTTCGTTCCAGCCCGAGGACGGCAGGCTC 3420  
A R V P F S A M D R W S F Q P E D G R L

3421 GCCCACGAGTCCGGGCGCTTCTTCTCCATCGAGGGCCCTGCACGTGCGGACGAACCTCGGC 3480  
A H E S G R F F S I E G L H V R T N F G

3481 TGGCGGCGGGAAGTTCGATCCAGCCCATCATCGTGACCCGAGATCGGCTTCTCGGCCTC 3540  
W R R D W I Q P I I V Q P E I G F L G L

3541 ATCGTCAAGGAGTTCGACGGTGTGCTGCACGTGCTGGCGCAGGCCAAGGCCGAGCCGGC 3600  
I V K E F D G V L H V L A Q A K A E P G

3601 AACATCAACGCCGTCAGCTCTCCCCGACCCTGCAGGCGACCCGACGCAACTACACCGGC 3660  
N I N A V Q L S P T L Q A T R S N Y T G

3661 GTCCACCGCGGCTCGAAGGTCCGGTTCATCGAGTACTTCAACGGCACGCGCCGAGCCGG 3720  
V H R G S K V R F I E Y F N G T R P S R

3721 ATCCTCGTCGACGTGCTCCAGTCCGAGCAGGGCGCGTGGTTCCTGCGCAAGCGCAACCGG 3780  
I L V D V L Q S E Q G A W F L R K R N R

FIG. 4B-4

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3781 AACATGGTCGTCGAGGTGTTTCGACGACCTGCCCGAGCACCCGAACCTCCGGTGGCTGACC 3840  
N M V V E V F D D L P E H P N F R W L T

3841 GTCGCGCAGCTGCGGGCGATGCTGCACCACGACAACGTGGTGAACATGGACCTGCGCACC 3900  
V A Q L R A M L H H D N V V N M D L R T

3901 GTGCTGGCCTGCGTCCCGACCGCCGTGGAGCGGGACCGGGCCGACGACGTGCTCGCGCGC 3960  
V L A C V P T A V E R D R A D D V L A R

3961 CTGCCCAGGGCTCGTTCCAGGCCCGGCTGCTGCACTCGTTCATCGGCGCGGGCACCCTG 4020  
L P E G S F Q A R L L H S F I G A G T P

4021 GCCAACAAACATGAACAGCCTGCTGAGCTGGATCTCCGACGTGCGCGCCAGGCCGCGAGTTC 4080  
A N N M N S L L S W I S D V R A R R E F

4081 GTGCAGCGCGGCCCGCCGCTGCCCGACATCGAGCGCAGCGGGTGGATCCGCCGCGACGAC 4140  
V Q R G R P L P D I E R S G W I R R D D

4141 GGCATCGAGCAGGAGGAGAAGAAGTACTTCGACGTCTTCGGCGTCACGGTGGCGACCAGC 4200  
G I E H E E K K Y F D V F G V T V A T S

4201 GACCGCGAGGTCAACTCGTGGATGCAGCGCTGCTCTCGCCCGCCAACAACGGCCTGCTC 4260  
D R E V N S W M Q P L L S P A N N G L L

4261 GCCCTGCTGGTCAAGGACATCGGCGGCACGTTGCACGCGCTCGTGCAGCTGCGCACCGAG 4320  
A L L V K D I G G T L H A L V Q L R T E

4321 GCGGGCGGGATGGACGTGCGCGAGCTGGCGCCTACGGTGCAGTGCAGCCCGACAACACTAC 4380  
A G G M D V A E L A P T V H C Q P D N Y

4381 GCCGACGCGCCCGAGGAGTTCGACCGGCGCTATGTGGACTACGTGTTGAACGTGCCGCGC 4440  
A D A P E E F R P A Y V D Y V L N V P R

4441 TCGCAGGTCCGCTACGACGCATGGCACTCCGAGGAGGGCGGCGGTTCTACCGCAACGAG 4500  
S Q V R Y D A W H S E E G G R F Y R N E

4501 AACCGGTACATGCTGATCGAGGTGCCCGCGACTTCGACGCCAGTGCCGCTCCCGACCAC 4560  
N R Y M L I E V P A D F D A S A A P D H

4561 CGGTGGATGACCTTCGACCAGATCACCTACCTGCTCGGGCACAGCCACTACGTCAACATC 4620  
R W M T F D Q I T Y L L G H S H Y V N I

4621 CACGTGCGCAGCATCATCGGTGCGCCTCGGCCGCTACACCAGGACCGCGGATGAAC 4680  
H V R S I I A C A S A V Y T R T A G \*  
M K R

4681 GCGCGCTGACCGACCTGGCGATCTCGGCGGCCCGAGGCATTCCTGCACACCTCTACG 4740  
A L T D L A I F G G P E A F L H T L Y V

FIG. 4B-5

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4741 TGGGCAGGCCGACCGTCGGGGACCGGGAGCGGTTCTTCGCCCGCCTGGAGTGGGCGCTGA 4800  
G R P T V G D R E R F F A R L E W A L N

4801 ACAACAACCTGGCTGACCAACGGCGGACCACTGGTGCGCGAGTTCGAGGGCCGGGTGCGCCG 4860  
N N W L T N G G P L V R E F E G R V A D

4861 ACCTGGCGGGTGTCCGCCACTGCGTGGCCACCTGCAACGCGACGGTCGCGCTGCAACTGG 4920  
L A G V R H C V A T C N A T V A L Q L V

4921 TGCTGCGCGCGAGCGACGTGTCCGGCGAGGTCGTATGCCTTCGATGACGTTTCGCGGCCA 4980  
L R A S D V S G E V V M P S M T F A A T

4981 CCGCGCACGCGGCGAGCTGGCTGGGGCTGGAACCGGTGTTCTGCGACGTGGACCCCGAGA 5040  
A H A A S W L G L E P V F C D V D P E T

5041 CCGGCCTGCTCGACCCCGAGCACGTGCGGTGCTGGTCAACCGCGGACGGGCGCGATCA 5100  
G L L D P E H V A S L V T P R T G A I I

5101 TCGGCGTGACCTCTGGGGCAGGCCCGCTCCGGTCGAGGCGCTGGAGAAGATCGCCGCCG 5160  
G V H L W G R P A P V E A L E K I A A E

5161 AGCACCAGGTCAAACCTCTTCTTCGACGCGCGCACGCGCTGGGCTGCACCGCGCGGGC 5220  
H Q V K L F F D A A H A L G C T A G G R

5221 GGCCGGTCGGCGCCTTCGGCAACGCCGAGGTGTTTCAGCTTCCACGCCACGAAGGCGGTCA 5280  
P V G A F G N A E V F S F H A T K A V T

5281 CCTCGTTCGAGGGCGGCGCCATCGTCACCGACGACGGGCTGCTGGCCGACCGCATCCGG 5340  
S F E G G A I V T D D G L L A D R I R A

5341 CCATGCACAACCTTCGGGATCGCACCGGACAAGCTGGTGACCGATGTCGGCACCAACGGCA 5400  
M H N F G I A P D K L V T D V G T N G K

5401 AGATGAGCGAGTGCGCCCGGGCGATGGGCTCACCTCGCTCGACGCCTTCGCCGAGACCA 5460  
M S E C A A A M G L T S L D A F A E T R

5461 GGGTGCACAACCGCCTCAACACGCGCTCTACTCCGACGAGCTCCGCGACGTGCGCGGCA 5520  
V H N R L N H A L Y S D E L R D V R G I

5521 TATCCGTGCACGCGTTTCGATCCTGGCGAGCAGAACAACTACCAGTACGTGATCATCTCGG 5580  
S V H A F D P G E Q N N Y Q Y V I I S V

5581 TGGACTCCGCGGCCACCGGCATCGACCGGACCAAGTTGCAAGGCGATCCTGCGAGCGGAGA 5640  
D S A A T G I D R D Q L Q A I L R A E K

5641 AGGTTGTGGCACAACCTACTTCTCCCCGGGTGCCACCAGATGCAGCCGTACCGGACCG 5700  
V V A Q P Y F S P G C H Q M Q P Y R T E

FIG. 4B-6

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5701 AGCCGCGCTGCGGCTGGAGAACACCGAACAGCTCTCCGACCGGGTGCTCGCGCTGCCCA 5760  
P P L R L E N T E Q L S D R V L A L P T

5761 CCGGCCCCGCGGTGTCCAGCGAGGACATCCGGCGGGTGTCGACATCATCCGGCTCGCCG 5820  
G P A V S S E D I R R V C D I I R L A A

5821 CCACCAGCGGCGAGCTGATCAACGCGCAATGGGACCAGAGGACGCGCAACGGTTCGTGAC 5880  
T S G E L I N A Q W D Q R T R N G S \*

5881 GACCTGCGCCACAAGTGCCAGGAGGTTCCGCTCCCCGATGAACACAACCTCGTACGGCAACC 5940  
M N T T R T A T

5941 GCCCAGGAAGCGGGGGTCCGCGACGCGGCGCGCCCGGACGTCGACCGGCGGGCGGTCTGTG 6000  
A Q E A G V A D A A R P D V D R R A V V

6001 CGGGCGCTGAGCTCGGAGGTCTCCCGCGTCACCGGCGCGGGTGACGGTGACGCCCACGTG 6060  
R A L S S E V S R V T G A G D G D A H V

6061 CAGGCGCGCCGGCTCGCCGACCTCGCCGCGCACTACGGGGCGCACCCGTTACGCGCGCTG 6120  
Q A A R L A D L A A H Y G A H P F T P L

6121 GAGCAGACGCGTGCGCGGCTCGGCCTGGACCGCGGAGTTCGCCCACCTGCTCGACCTG 6180  
E Q T R A R L G L D R A E F A H L L D L

6181 TTCGGCCGATCCCGGACCTGGGCACCGCGGTGGAGCACGGTCCGGCGGGCAAGTACTGG 6240  
F G R I P D L G T A V E H G P A G K Y W

6241 TCCAACACGATCAAGCCGCTGGACGCCGCGGCGCACTGGACGCGCGGTCTACCGCAAG 6300  
S N T I K P L D A A G A L D A A V Y R K

6301 CCTGCCTTCCCTACAGCGTCCGGCTGTACCCCGGGCGGACGTGCATGTTCCGCTGCCAC 6360  
P A F P Y S V G L Y P G P T C M F R C H

6361 TTCTGCGTGCGGGTGACCGGTGCCCGCTACGAGGCCGATCGGTCCCGGCGGGCAACGAG 6420  
F C V R V T G A R Y E A A S V P A G N E

6421 ACGCTGGCCGCGATCATCGACGAGGTGCCACGGACAACCCGAAGCGGATGTACATGTGG 6480  
T L A A I I D E V P T D N P K A M Y M S

6481 GGCGGGCTCGAGCCGCTGACCAACCCCGGTCTCGGCGAGCTGGTGTGCGACGCCCGCGGG 6540  
G G L E P L T N P G L G E L V S H A A G

6541 CGCGGTTTCGACCTCACCCTCTACACCAACGCCTTCGCCCTCACCAGCAGACGCTGAAC 6600  
R G F D L T V Y T N A F A L T E Q T L N

6601 CGCCAGCCCGCCTGTGGGAGCTGGGCGGATCCGCACGTCCCTCTACGGGCTGAACAAAC 6660  
R Q P G L W E L G A I R T S L Y G L N N

FIG. 4B-7



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6661 GACGAGTACGAGACGAUCCACCGGCAAGCGGGCGCTTTCGAACGCGTCAAGAAGAACCTG 6720  
D E Y E T T T G K R G A F E R V K K N L

6721 CAGGGCTTCCTGCGGATGCGCGCCGAGCGGGACGCGCCGATCCGGCTCGGCTTCAACCAC 6780  
Q G F L R M R A E R D A P I R L G F N H

6781 ATCATCCTGCCGGGACGGGCGGACCGGCTCACCGACCTCGTCGACTTCATCGCCGAGCTC 6840  
I I L P G R A D R L T D L V D F I A E L

6841 AACGAGTCCAGCCCGCAACGGCCGCTGGACTTCGTGACGGTGCGCGAGGACTACAGCGGC 6900  
N E S S P Q R P L D F V T V R E D Y S G

6901 CGCGACGACGGCCGGCTGTCCGACTCCGAGCGCAACGAGCTGCGCGAGGGCCTGGTGCGG 6960  
R D D G R L S D S E R N E L R E G L V R

6961 TTCGTCGACTACGCCGCGGAGCGGACCCCGGGCATGCACATCGACCTGGGCTACGCCCTG 7020  
F V D Y A A E R T P G M H I D L G Y A L

7021 GAGAGCCTGCGGCGGGGTGTGGACGCCGAGCTGCTGCGCATCCGGCCGGAGACGATGCGT 7080  
E S L R R G V D A E L L R I R P E T M R

7081 CCCACCGCGCACCCCCAGGTCCGGTGCAGATCGACCTGCTCGGCGACGTCTACCTCTAC 7140  
P T A H P Q V A V Q I D L L G D V Y L Y

7141 CGCGAGGCGGGCTTCCCGGAGCTGGAGGGCGCCACCCGCTACATCGCGGGCCGGGTCACC 7200  
R E A G F P E L E G A T R Y I A G R V T

7201 CCGTCGACCAGCCTGCCGAGGTGGTGGAGAACTTCGTGCTGGAGAACGAGGGCGTGCGAG 7260  
P S T S L R E V V E N F V L E N E G V Q

7261 CCCC GCCCGGCGACGAGTACTTCTCGACGGCTTCGACCAGTCGGTGACCGCACGGCTC 7320  
P R P G D E Y F L D G F D Q S V T A R L

7321 AACCAGCTCGAACGAGACATCGCCGACGGGTGGGAGGACACCGCGGCTTCTGCGCGGA 7380  
N Q L E R D I A D G W E D H R G F L R G

7381 AGGTGAACCGAGTTGCGAGTACGTGAGCTGGCGGTGGCGGGCGGTTTCGAGTTCACCCC 7440  
R \* V A G G F E F T P

7441 CGACCCGAAGCAGGACCGGGGGGCTGTTCGTGTCTCCGCTGCAGGACGAGGCGTTCGT 7500  
D P K Q D R R G L F V S P L Q D E A F V

7501 GGGCGCGGTGGGCCATCGGTTCCCGTCGCCAGATGAACACATCGTCTCCGCCCGGGG 7560  
G A V G H R F P V A Q M N H I V S A R G

7561 CGTGCTGCGCGGGCTGCACTTCAACCAACCCCGCGGGGCGAGTGCAAGTACGTCTACTG 7620  
V L R G L H F T T T P P G Q C K Y V Y C

FIG. 4B-8

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7621 CGCGCGCGGCGGGCGCTCGACGTCATCGTCGACATCCGGGTCGGCTCGCCGACGTTCCGG 7680  
A R G R A L D V I V D I R V G S P T F G

7681 GAAGTGGGACGCGGTGGAGATGGACACCGAGCACTTCCGGGCGGTCTACTTCCCCAGGGG 7740  
K W D A V E M D T E H F R A V Y F P R G

7741 CACCGCGCACGCCTTCTCGCGCTTGAGGACGACACCCTGATGTCGTACCTGGTCAGCAC 7800  
T A H A F L A L E D D T L M S Y L V S T

7801 GCCGTACGTGGCCGAGTACGAGCAGGCGATCGACCCGTTGACCCCGCGCTGGGTCTGCC 7860  
P Y V A E Y E Q A I D P F D P A L G L P

7861 GTGGCCCGCGGACCTGGAGGTCGTGCTCTCCGACCGCGACACGGTGGCCGTGGACCTGGA 7920  
W P A D L E V V L S D R D T V A V D L E

7921 GACCGCCAGGCGGCGAGGGATGCTGCCCGACTACGCCGACTGCCTCGGCGAGGAGCCCGC 7980  
T A R R R G M L P D Y A D C L G E E P A

7981 CAGCACCGGCAGGTGACGGGTCCCGAGCACGATCTGTTGGAAGTGGCGCAGGCGCTCGTC 8040  
S T G R \*

8041 GTCGCGGTCTGA 8051

FIG. 4B-9

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```

1      50
eryBIV vngidsprq litllGaSGf vGsavlreLr dhpv.rlrav sRggapavpp
ascF   .....mk. .llitGvSGY iGshlvnyLa nlggyeiYgi sRneildqdi
rfbJ   ....mtflke yvivsGaSGf iGkhllleaLk k.sgisvvai tRdvi..knn
strL   ...mspyprp rwlvtGaSGm lGreltPlld rrga.avtal gRghl.ditd
Consensus -----G-SG- -G-----L- -R-----

```

```

51      100
eryBIV gaaevedlra dllepgraaa aiedadvivh lvahaAggst wrsatsdpe.
ascF   nql.llniki fqldrdsldp ilkrvrpdv. .vihlAscfl sqhsyknike
rfbJ   sna.lanvrw cswdniellv eelsidsali giihlAtey. .ghktsslin
strL   gaa..... . ....vrsav aehrpavvn caawtAvd.. ..eaesepal
Consensus -----A-----

```

```

101     150
eryBIV aervNvglmh dLvgalhdr rstopvllly staqaanpsa asryaqqkte
ascF   iiksNvefpt eLlea.....mndv gvkkiintgt swqcfnsdty
rfbJ   iedanvikpl kLldl.....aiky radiflntd. sffakkdfny
strL   amavNgegpr hLaqa.....c ravgavllql stdyvfpgsg grpyredhpt
Consensus ----N-----L-----

```

FIG. 5A-1

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151	eryBIV	aerilrkatd	egrvrgvilr	lpa.....	..vygqsGps	gpmgrgvvaa	200
	ascF	npvnlyaask	qafedilkfy	inaegfsain	lklfdtyGgv	dkrrklis.l	
	rfbJ	qhmrpyiitk	rhfdeighyy	anmhdifvn	mrlehvyGpg	dgenkfipyi	
	strL	gprrtyvgctk	rageravlev	lpdtgyivrt	awlygagGp.	.....nfvak	
	Consensus	-----	-----	-----	-----G-	-----	
201	eryBIV	mirralagep	ltmwhdggvr	rdl.lhveDv	atAFaaaleh	hdalag...g	250
	ascF	lddiaknnkq	ldmspgeqll	d..lvhindv	craFkiaidk	lcelpseyvv	
	rfbJ	idclnkkqsc	vkcttgeqir	d..fifvdDv	vnAYltilen	rkevps..yt	
	strL	mirleadedt	vlvddqhgq	ptwtadlaDr	laALgaaala	gtapagiyha	
	Consensus	-----	-----	-----D-	--AF-----	-----	

FIG. 5A-2

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```

300
eryBIV      twalgadrse plgDifravS gsVa..rqtg spavdvvtvp apehaeandf
ascF        sygvsnkyrv tlkElvsiye ..rv...nnv klninfgtre yrnrevmvpC
rfbJ        eyqvgtgagv slkDflvylq ntmm...pgs ssifefgaie qrdneimfsv
strL        tntggttwna lapEtfrllg adparvrptt slalarpavr .prysvldqs
Consensus  -----

```

```

301
eryBIV      rsddidstef rsrtgwrprv sltdgidrtv aaltpteeh .....
ascF        .tniqnl... ..pdwevvi plsqglky.. .....
rfbJ        .annknlkam gwkpndfykk gieellkrl. ....
strL        rwkaaglepl rhw.....ra altesf.... palcgragrp vpgpr
Consensus  -----
345

```

FIG. 5A-3

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```

1      50
eryBVII  ....      .....Vagg feftdpkqD rRGlFvsplQ deaFvgavGh
strM.    ..      .vrplsVqga wlsetrafAD dRGefqelys arslrgalGY
rfbC     .....vm      iviktaIpdv lilpkvfgD eRGfFfesYn qqtFeeliGr
rfbF     .....m      kctklsIpev ilfeprifED dRGhFfesFn lakFqesiGr
asCE     .....m      ifkkldiegc yliefnkfid sRGtFvktFh sdfFsen.Gi
Consensus -----I-----D -RG-F-----F-----G-

```

```

51      100
eryBVII  rfpvaqmnhI vSargVlRGl HFtttppgqc KyVycarGra LDVivDiRvG
strM     dpgvaqvnrS vSrrgVlRGV Hfaqlppsqa KyVtclsGav LDVvvdirtg
rfbC     kvtfvqdnhs kSkknVlRGl HFqrqenaqg KlVrcavGev FDVavDiRke
rfbF     qvtfvqsnes ySkqnViRGl HYq.virpqq KlVrvveGev FDIavDiRks
asCE     vldmreefys iSaknViRgm HFqmppaehd KlVycvnGav LDVilDiRkd
Consensus -----S---V-RG-- HF-----K-V-----G-- LDV--D-R--

```

```

101      150
eryBVII  SpTFgkWdAV emdtehfrav YfprGtaHaF laLeddtlms Ylvstpyvae
strM     SpTYraWeav rLddph.rsl YveaGlghsF maLttddavv Yltsggyaag
rfbC     SpTFgqWvgV nLsaenkrql WipeGfaHgF vtLseyaeFl Ykatnyysps
rfbF     SpTFgqWvgV lLsdknqhql WipeGfgHgF qvLspsakfq Ymvtidywype
asCE     skTYgeYfsI eLsyenslal WvpkGlaHgF lsladnsimf Yktssvhnve
Consensus S-TF--W--V -L-----W---G--H-F --L-----Y-----

```

FIG. 5B-1

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eryBVII	151	yEgaIdpfdp	200	gmIpdyacl
strM		rEhgVhpldp		gllpdyeecl
rfbC		sEgsIlwnde		aplldqallt
rfbF		hDrcIrfnds		...lsakdaa
asCE		cDsgIkwnsf		eyklsskdis
Consensus		-E--I-----		f.....
				-----E-----
eryBVII	201	geepastgr	212	
strM		afrrslcerg		tg
rfbC		e.....		
rfbF		.....		
asCE		.....		
Consensus		-----		

FIG. 5B-2

FIG. 5C-1

1	50
eryCIV	.....mk raltDLaifg gpeaFlhtly vgrptVgd.. ....rerFfa
eryCI	.....md vpfldLqa.. ayleLrsdid qAcrrVlg.. ....sgwY..
ascC	...msqeelr qqiaelVaq. aetaMapkpf eAgksVvpps gkvigtKelq
dnrJ	..... vstyvWqylN eyreeradiL dAveTVfe.. ....sgqL..
prgI	.....
strS	.....mssFqelp rwpqLtdddi eAavaalr.. ....snrL..
Consensus	-----L-----A-----V-----
51	100
eryCIV	rlewalnnnw Ltnggplvre FEgrvAdL.. aGvrHcVatc natvAlqLvL
eryCI	..... Lhgpe..nea FEaeFAaY.. cenaHcVtvg SGcdAleLsL
ascC	lmveasldgw L.ttgrfnda FEkkLgeYl. .Gvpyvlttt SGssAnlLAL
dnrJ	..... ilgts..vrs FEeeFAaY.. hGlpyctgvd nGtnAlvLgL
prgI	..... .sgp..igq LEaeFlaFlD hGvryaVtfn SGtsAlLaAY
strS	..... vgggnstvee FEaaLaa..g qGveHaVavs tGtaAvhLAL
Consensus	-----L-----FE-----A-Y-- -G--H-V-- SG--A--LAL
101	150
eryCIV	rAs..... .dv..sgEVV mpsmTFaaTa haaswlGleP VFcdVDpEtg
eryCI	VAL..... .gVgqGDEVI vPshTFiaTw lGv.pvGAvP VpVEpEgVsh
ascC	tALtsPklgv ralkPGDEVI tvaagFptTV nptiqnGlIP VFVDVDipt.
dnrJ	rAL..... .gIgPGDEVV tvsnTaaptV vaIdavGATP VFVDVhe....
prgI	fAL..... .gVreGvEaa gPaLTYhaal spVfalrgdv VLVDIdpvsr
strS	hAL..... .dvGPGDEVI vPthTFigsa spVtylGARp VFAdVtpdTh
Consensus	-AL----- --V-PGDEVI -P--TF--T- --V---GA-P VFVDVD--T-



FIG. 5C-2

151	eryCIV	.....	1LDP	ehVaslVTpr	TgAigVHlW	GrpapVeale	kIAaeHgvkl	200
	eryCI	t.....	LDP	alVEqAITpr	TaAilpVHlY	GhpaDLdalr	aIAdRHgLaI	
	ascC	.....	ynvna	sLIEAAVsDk	TkAImiaHtI	GnlfDLaeVr	rVAdkynLwI	
	dnrJ	...enylMDt	grlrsVlgpr	TrcllpVHlY	GqsvDMtpvI	eIAaeHdLkV		
	prgI	g.....	LDP	kaleAAITen	TrvVtvVHqW	GhpcDMDaIl	gVAerygLvV	
	strS	c.....	LDP	dsVksliGEr	TkAIvvVHIn	GiaaDMAalt	aVAaeagvpV	
	Consensus	-----	LDP	--VEAAITE-	T-AI--VH-Y	G---DMD-V-	-IA--H-L-V	
								250
	eryCIV		201	ffDaAhAlGc	tagGrpVGaF	GnaevFS.Fh	atKavtsf.E	GGAIVTdDgll
	eryCI			VEDvAQAVGa	rhrGhrVGag	snaaafS.FY	PgKnlgALGD	GGAVVtTDpal
	ascC			IEDccdAlGs	tydGkmaGtF	GdigitVS.FY	Pahhitm.GE	GGAVftQsael
	dnrJ			IEDcAQAhGa	rrhGrIvGtq	GhaaaFS.FY	PtKvlgAYGD	GGAVVtPdaev
	prgI			IEDcshAhGs	rYkGkvpGtF	GdaavFS.Lq	anKavyA.GE	GgiIvTDdalv
	strS			IEDaAQAlGt	eigGrPIgGf	GdlacvSlFF	eqKvitsgGE	GGAVITdnpyv
	Consensus			IED-AQA-G-	-Y-G--VG-F	G----FS-FY	P-K---A-GE	GGAVVT-D--L
								300
	eryCIV		251	aDRiRaMhn	FGiapdk...	.....	.....	lvtadvG
	eryCI			aERiRLRn	YG.....sk.	.....	.....	qk.Yvhevrg
	ascC			ksiiiesFRd	WGrdcycapg	cdntckkrfg	qqlgslpfgy	dhkYtyshlg
	dnrJ			drRLrRLRY	YG.....mg.	.....	.....	eryYvvdtpG
	prgI			qDRatlLat	tG.....t..	.....	.....	.....vpg
	strS			aERvRrLRs	hGegpvsGs.	.....	.....	pgmiwahevG
	Consensus			-ER-R-LR-	YG-----	-----	-----	--Y-----G

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```

301          tNgkMsEcaA  AmgltsLdaF  aetrvhnrln  halysdeLrd  vrGisvhafd
eryCIV      tNaRLdELQA  AvlrvkLrhL  DdWnarRttl  aghyqteLkd  vpGitlpeth
eryCI       yNikitDMQA  AcglaqLepi  EeFvekrkan  fkyldalQs  cadf.ielpe
ascC        hNsRLdEvQA  eilrrkLrrL  DaYvegRrav  arryeegLgd  ldGlvlpt...
dnrJ        lghRr.....
prg1        yNvRLtsvQA  psaspsnkrL  gdLveaRrrn  aaylserLag  veGlelpvpep
strS        -N-RL-E-QA  A-----L--L  D-W---R---  -----L--  --G-----
Consensus

```

```

351          pgeqnnyqYv  iisVdsaatg  idr...dqlQa  iLraekVvaq  pyFspgcHqm
eryCIV      pwads..aWh  lFvlrcenrD  .....hLqr  hLtdagVqt1  ihYtpvHls
eryCI       atensdpsWf  gFpI.tlked  sgvsridLvk  fLdeakVgtr  llFagnltrq
ascC        iaegn dhvYy  vYvVrhperD  .....rile  aLtaydiHln  isYpwpvHtm
dnrJ        .....
prg1        p...gtthaYw  kYaVrvvpGD  grrsaadiaa  hLrsrgVpvl  lrYpyplHkq
strS        -----W-  -Y-----D  -----L--  -L-----V---  --Y-----H--
Consensus

```

FIG. 5C-3

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eryCIV	401	qpYrte...p	plrLentEqL	sdrvLaLPtg	PavssEdirr	Vcdiirlaat	450
eryCI		payAdlgl.p	pGsFpvaEsL	agevLsLPig	PhLsrEaaDh	VIatlkaga	
ascC		pyFhdvkyRv	vGeltnDRI	mnqtFwigiy	PgLthDhLDy	VWskfeefig	
dnrJ		sgFAhlg.Yg	pGdLpvTERL	ageiFsLPmy	PsLrpDaqEk	VIdavrevvg	
prg1		.....	.....	.....	.....	.....	
strS		paFAe...Yh	gvsLpvaERL	sqellaLPsh	PgLveghLDh	aveevrkava	
Consensus		--FA-----Y-	-G-L---ERL	----L-LP--	P-L--E--D-	VW-----	
eryCIV	451	sgelinaqwd	qrtrngs				468
eryCI		.....	.....				
ascC		lnf.....	.....				
dnrJ		sl.....	.....				
prg1		.....	.....				
strS		s.....	.....				
Consensus		-----	-----				

FIG. 5C-4

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1	eryBV	vrVlltsfah	rtHFqGLVPL	AWALRTAGHD	VRVaaQPALT	DaVigAGLTA	50
	eryCIII	mrVvfssmas	kSHLfgLVPL	AWAFRaAGHE	VRVvasPALT	EditaAGLTA	
	dnrS	mkVlvtafam	daHFNgvVPL	AWALRaAGHD	VRVasQPALT	DsItrAGLTA	
	Consensus	--V-----A-	--HF-G-VPL	AWALR-AGHD	VRV---PALT	D-I--AGLTA	
51	eryBV	VPVGsDhrlf	divpevaaqV	hrysfyIDFY	hreqelhsWE	fLlgmqeats	100
	eryCIII	VPVGtDvdIv	dfmthaghdI	idyvrslDFS	erdpatlWE	hLrgmqtvlt	
	dnrS	VPVGtDhqvq	aamgamapGV	falhlnpDYL	enrpelldLE	fLeastsmlt	
	Consensus	VPVG-D----	-----V	-----DF-	-----E	-L-----	
101	eryBV	rwYpvmnd	sFvaeLVdFa	rdWRPDLVLW	EPFTFagava	aracGaahAR	150
	eryCIII	ptfYalmspd	tLIegMVsfC	rkWRPDLViW	EPLTfaapia	gavtGtphAR	
	dnrS	aafYaqinnd	sMIdemVdFa	awWRPDLVvW	EPFTFggava	aqvtGaaqAR	
	Consensus	---Y-----D	--I--MV-F-	--WRPDLV-W	EPFTF---VA	----G----AR	

**FIG. 5D-1**

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```

151          200
    eryBV    LLWGsDltgy frgrFqaql rrPpEdRpdLp LgtWLTevag rfGv.eFgeD
    eryCIII  LLWGPdIttr arqnFlgllp dqpeEhRegp LaeWLTwtle kyGgpaFdeE
    dnrS     LLWGPdLflr vhdrFqqvlh evPaErRdda LeeWLTwtle rhGa.aFgpe
    Consensus LLWG-D-----F----- --P-E-R--- L--WLT----- --G---F---E

250
    eryBV    lavGqWsVDq lPpsfRLdTG metVvartlp YNG..asVVP dWLkksatr
    eryCIII  vvvGqWtIDp apaaIRLdTG lktVgmRyvD YNG..psVVP eWLhdeper
    dnrS     visGhWtIDq mPpsvRFaTa rpTVpmRfvp YNGpvpavVP pWLradpgrp
    Consensus ---G-W-ID- -P----RL-T- --TV---R--- YNG-----VVP -WL-----

300
    eryBV    RiciTgGfsg lgla.adadq fartLaqlar fdGEIVvTgs gpdtsav...
    eryCIII  RVclTlGiss rensigqvs. ieellgavgd vDaEIIaTfd aqlegvani
    dnrS     RVllTqGite rstgftglpr agellasiae lDaEVVaTvk aeeregppl
    Consensus RV--T-G----L----- --L----- -D-EIV-T-- -----

```

FIG. 5D-2

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	350		
eryBV	PdNIRlVdfv	pMgvllQnCA	AIHHGGaGt WAtAlhHGIP QisvaheWDC
eryCIII	PhNVRtVgfv	pMhallptCA	AtVHHGGpGs WhTAaiHGVP QvllpdgWDC
dnrS	PgNVRvVds1	sLhvvLpsCA	AvVHHGGaGt WAtAalHGVP QlalaWqWDD
Consensus	P-NVR-V---	-M---L---CA	A-VHHGG-G- W-TA---HGVP Q-----WD-
	351		
eryBV	mLRgqqtAeL	GAGIyLrp..	devdadslas altqVvedPt ytenAvklRe
eryCIII	gvRaqrqtqef	GAGIaLp..v	peltpdqlre svkrVldDPa hragAarmRd
dnrS	vfRagqlekl	GAGIfLpphg	egasagrVrd rlaqVlaEPs frqgAariRa
Consensus	--R-----L	GAGI-L----	-----V---DP- ----A---R-
	401		
eryBV	EaLsdPtPqe	IVprlEeLtr	rhag.....
eryCIII	DmLaepSpae	VVgicEeLaa	grrepr.....
dnrS	EmLrtPaPga	VVptlEqLta	rhrapagqgv rh
Consensus	E-L--P-P--	VV---E-L--	-----
	433		

FIG. 5D-3

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1  
 eryCVI MYegg.faeI YDrfyrgRgK DYaaeaqva rlvrdrlpsA ssLLDVACGT 50  
 srmX MYendsaaEv YDllyqdr.K DYageaarvt dlirertpda asLLDIACGT  
 rdmD MYgad.lArv YDlvhreRgK DYrardrggr rrgpaeqagA grLLDVACGT  
 Consensus MY-----A-- YD-----R-K DY-----A --LLDVACGT

51  
 eryCVI GtHLrrFAdL FddvtGLELS aaMievArpq LgGlpvlqGD MRdFaLdref 100  
 srmX GtHLeaFAKL YdrVSGLELS ewMaarAeer LpGVtlhrGD MRaFdLgetF  
 rdmD GgHLrhFAdL FahVeGvELS epMaeeAraa LpGVtvhaGD MRdFrLgttF  
 Consensus G-HL--FA-L F--V-G-ELS --M---A--- L-GV-----GD MR-F-L---F

101  
 eryCVI DaVtCMFSSI GhMrdgAEld qAlasfARHL apgGVvvVEP WWFpEdFIDG 150  
 srmX DaVvCMFSSI GyLettADLe dAvaamARHL tadGVlaVEP WYFpDtFIDG  
 rdmD DvVtCMFgSV GyMtsvAElg rAlrmfARHL epgGVavVDP WWFyEtFaDG  
 Consensus D-V-CMF-SI G-M---AEL- -A-----ARHL ---GV---VEP WWF-E-F-DG

FIG. 5E-1

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```

151          yVagdvvd. .gdltIsRVS HsvRaGgatr MEIHWvVAda vnGprHhvEh
          hvsthalrta pgdqgVaRVS HstReGgrtr MEIHylIAht aeGirHrSEv
          hVsadi tv. .dgvTVsRVS HSaRrGrtsh MDVHFvVAep gaGaQHfvDt
          -V----- -V-RVS HS-R-G---- MEIH--VA-- --G--H--E-
          Consensus

```

```

201          yeitLFerGg YEKAftaAGc avqYleggps grGLFvGvRg
          srmX dyltLFsRae YEaAYrkAGl dVeYvvtgeg spGFFlGtrr
          rdmD hiisLFsRse YEqAFrdAGf aveYlpeaps grGLFvGvRg
          Consensus ----LF-R-- YE-AF--AG- -V-Y----- --GLF-G-R- -

```

FIG. 5E-2



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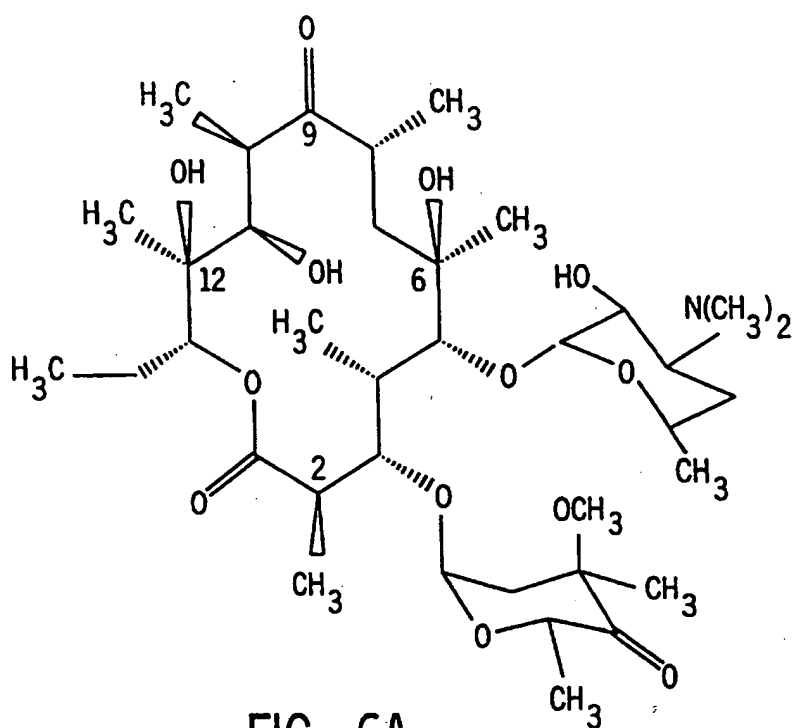


FIG. 6A

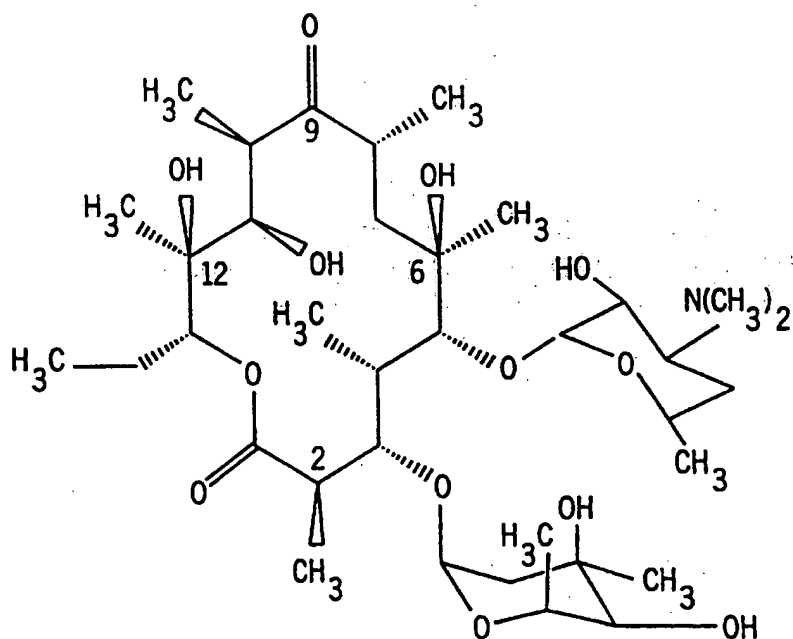


FIG. 6B

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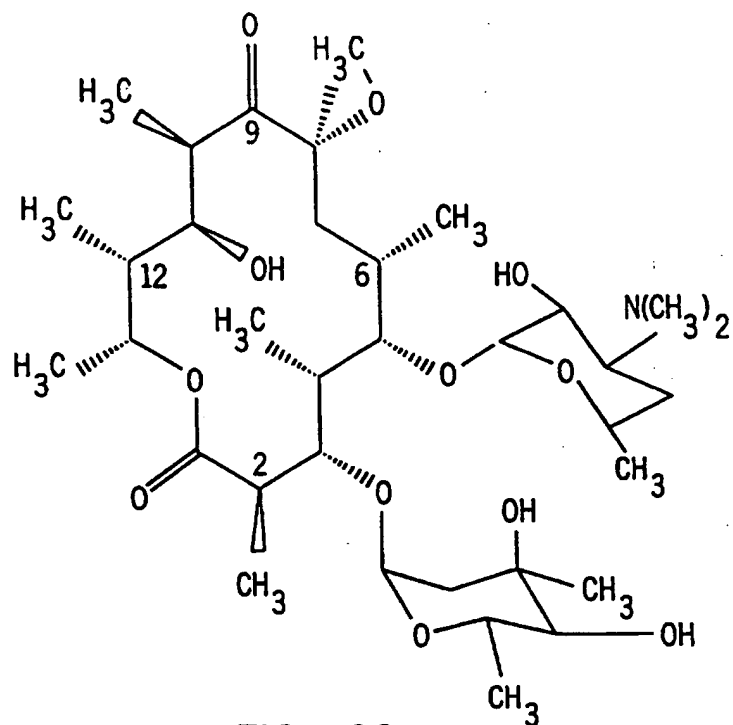


FIG. 6C

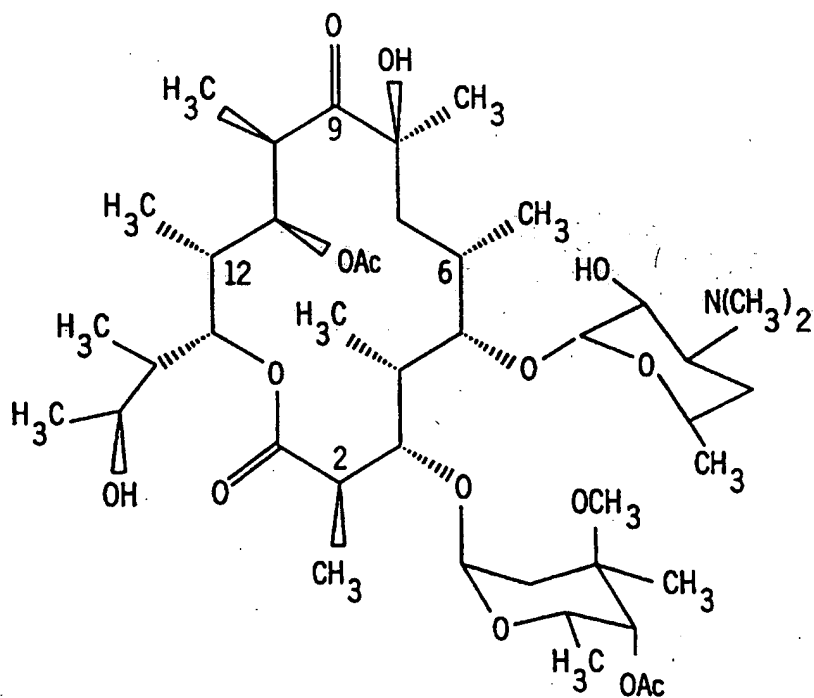


FIG. 6D

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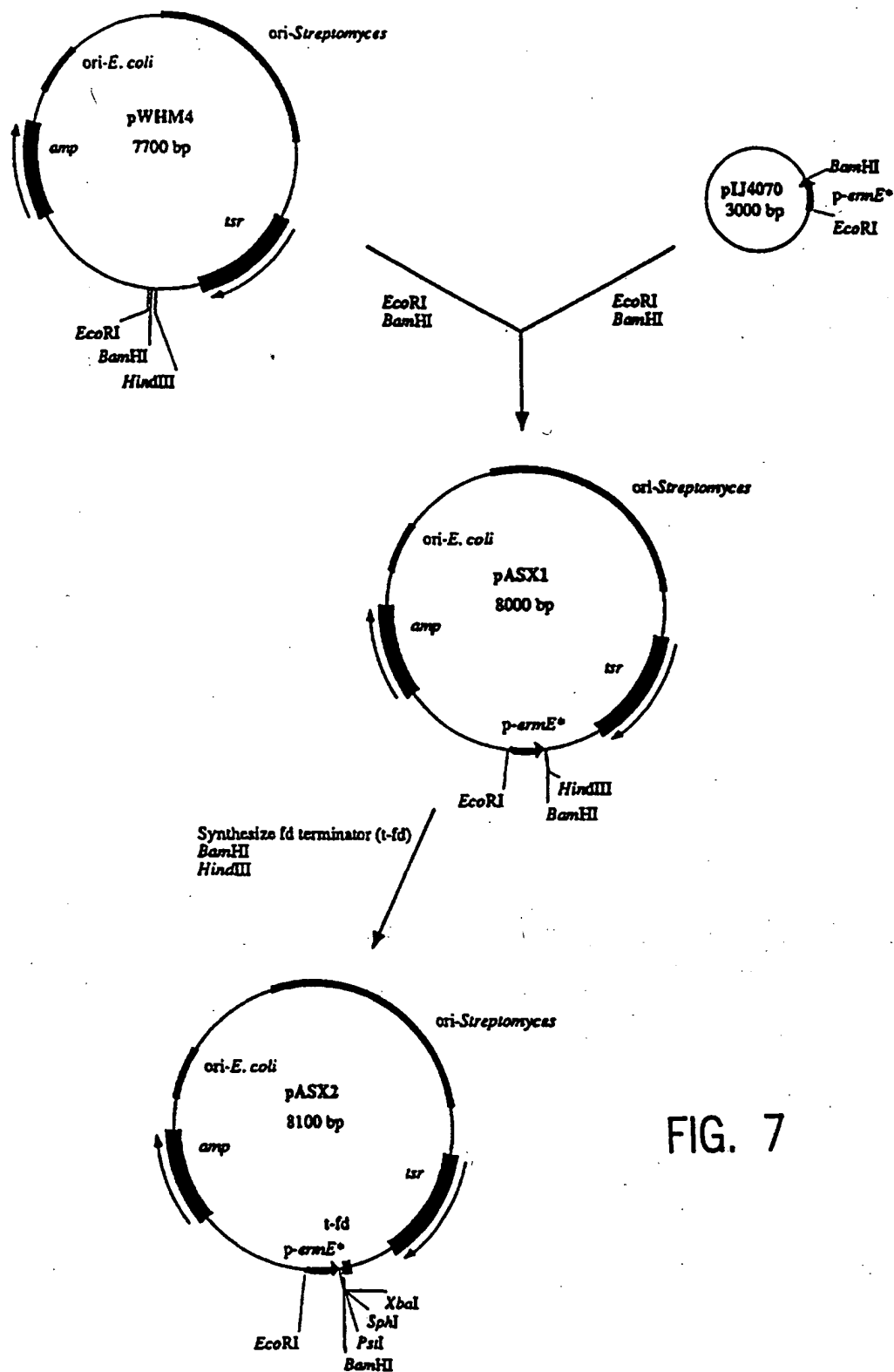


FIG. 7

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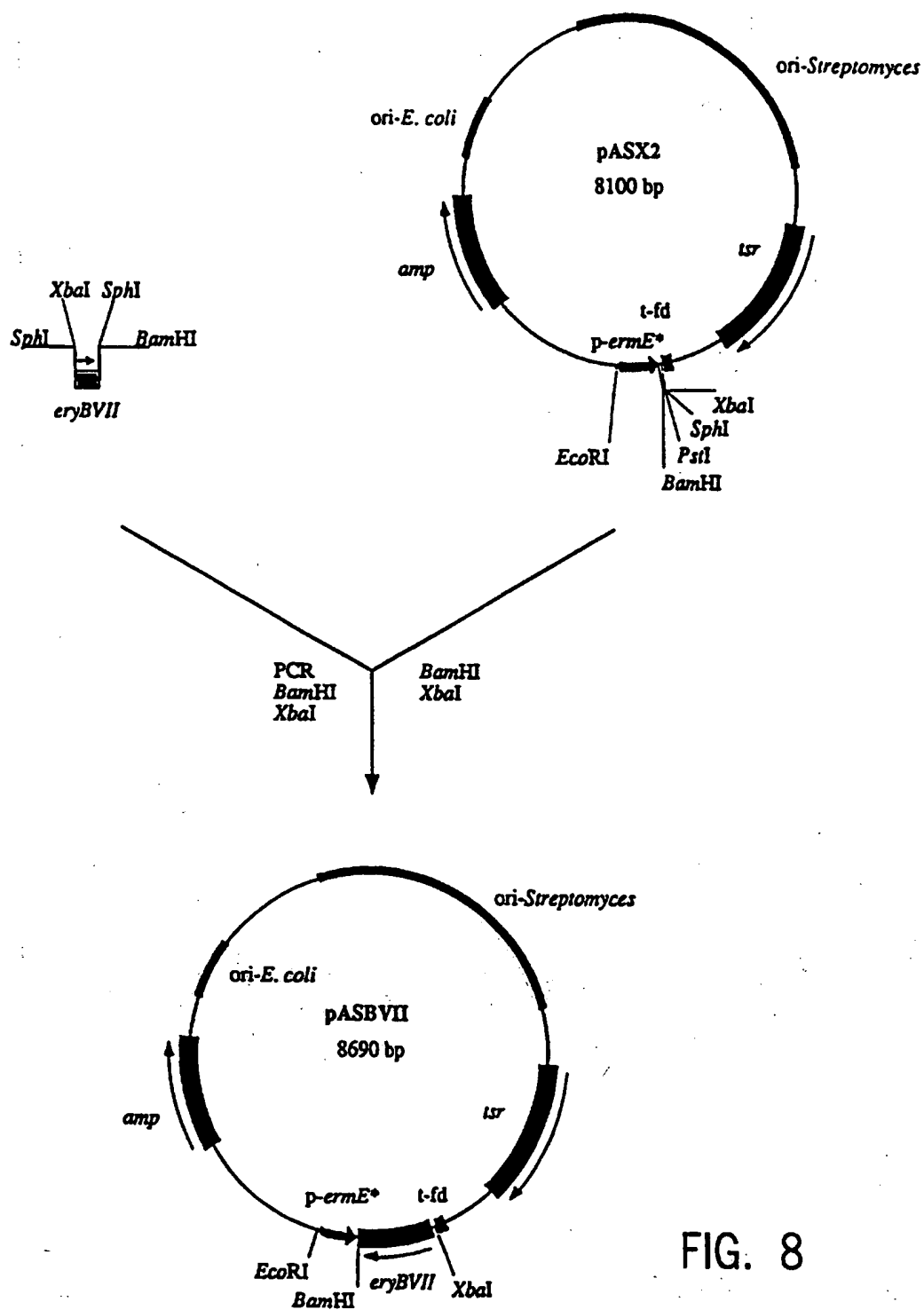


FIG. 8

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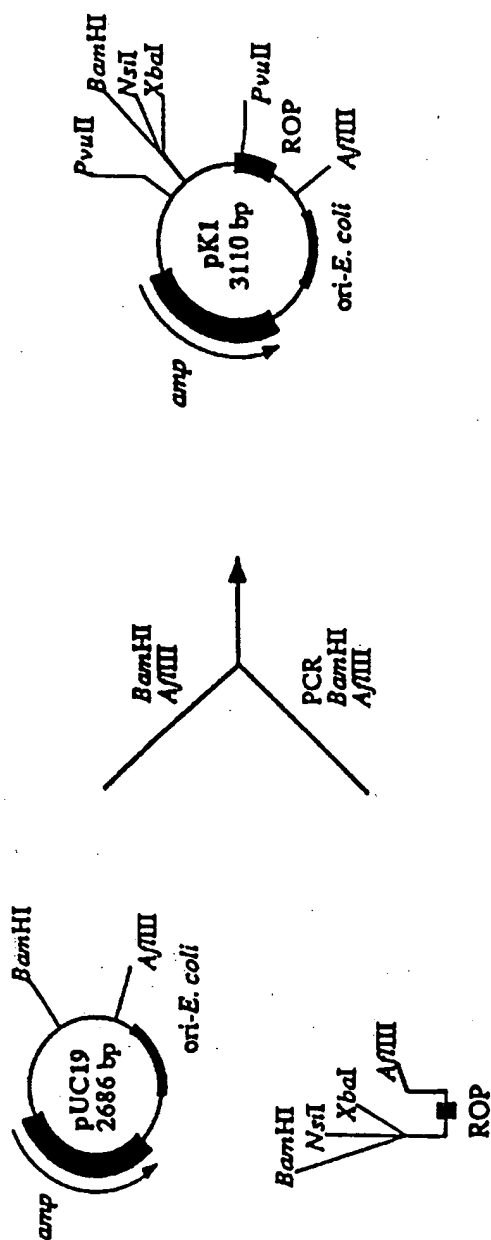


FIG. 9A

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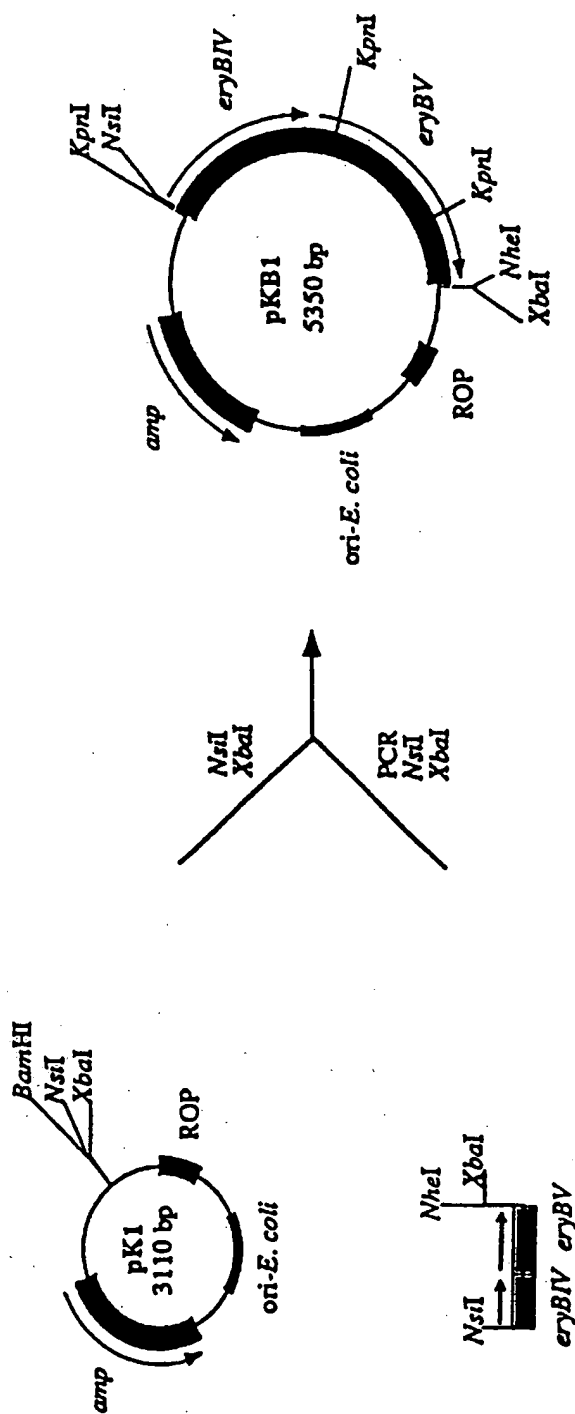


FIG. 9B

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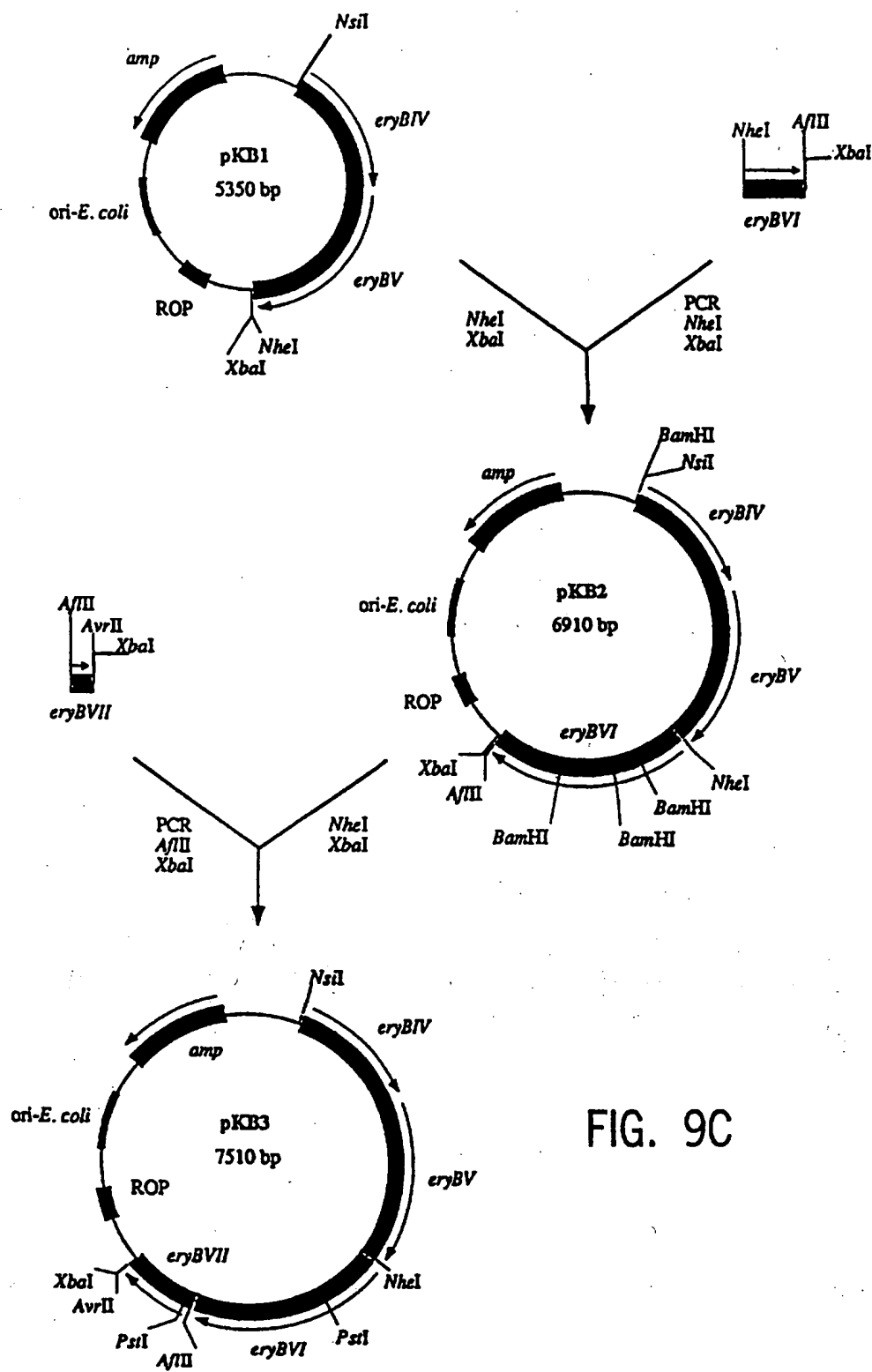


FIG. 9C

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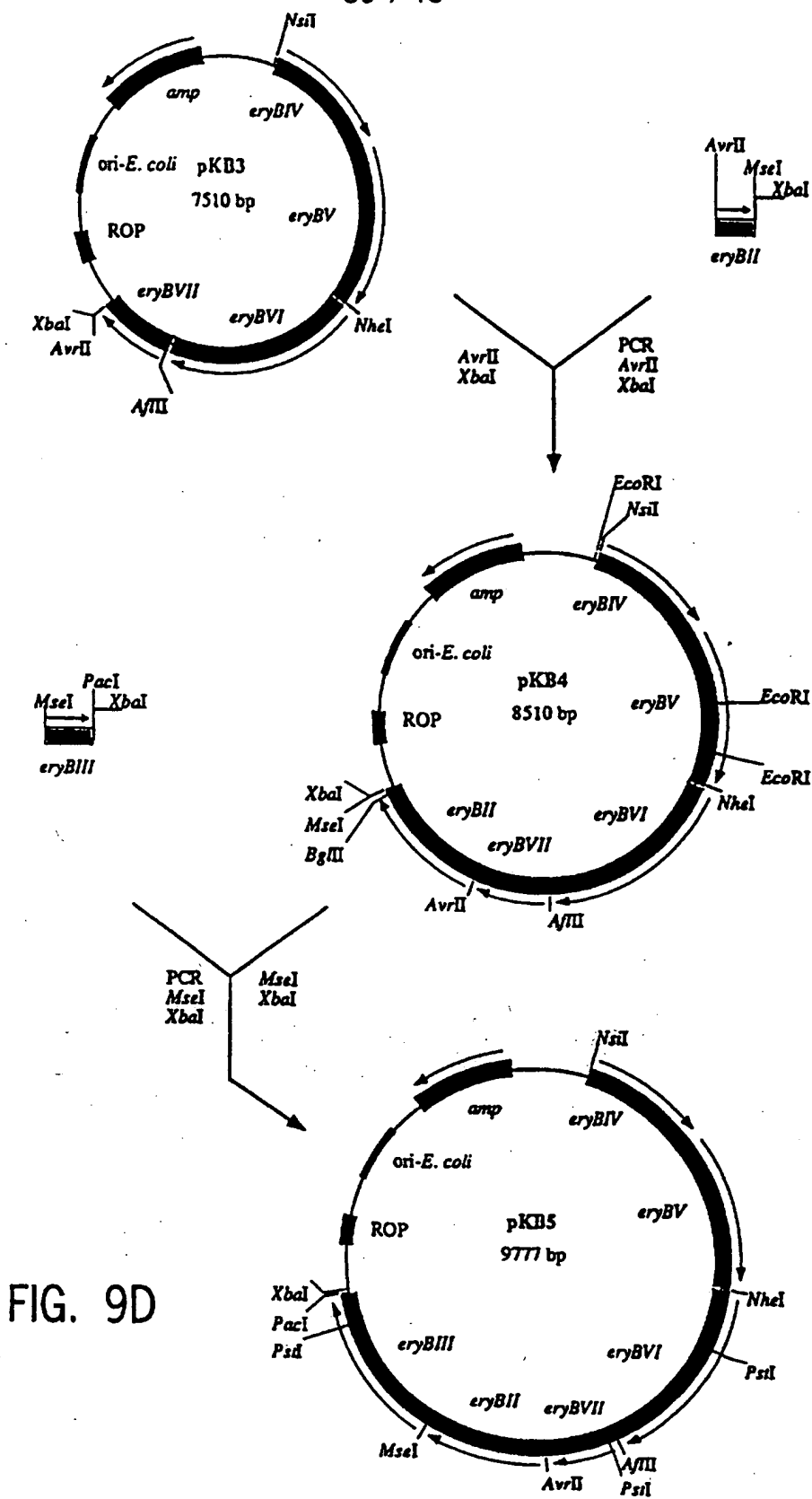


FIG. 9D



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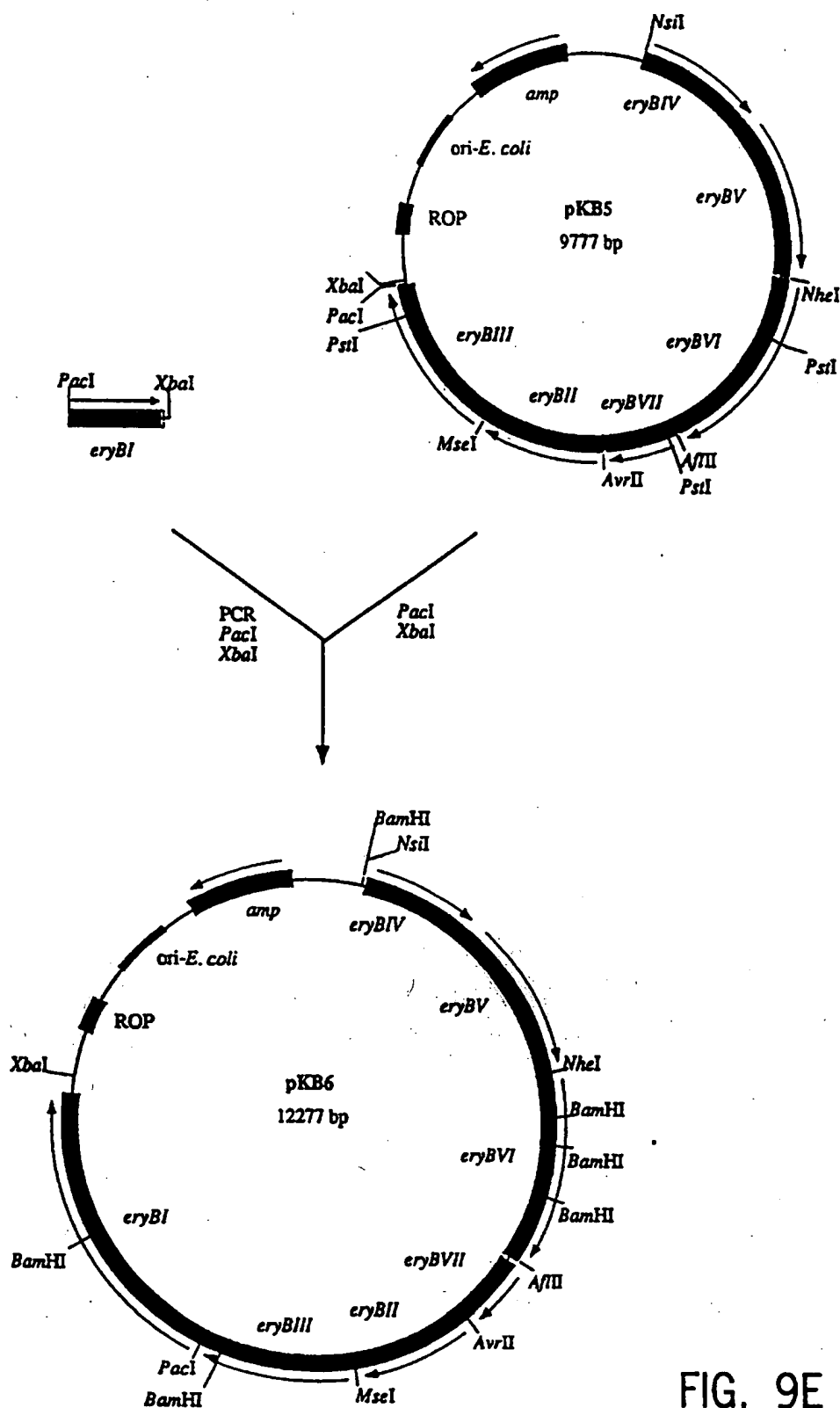


FIG. 9E

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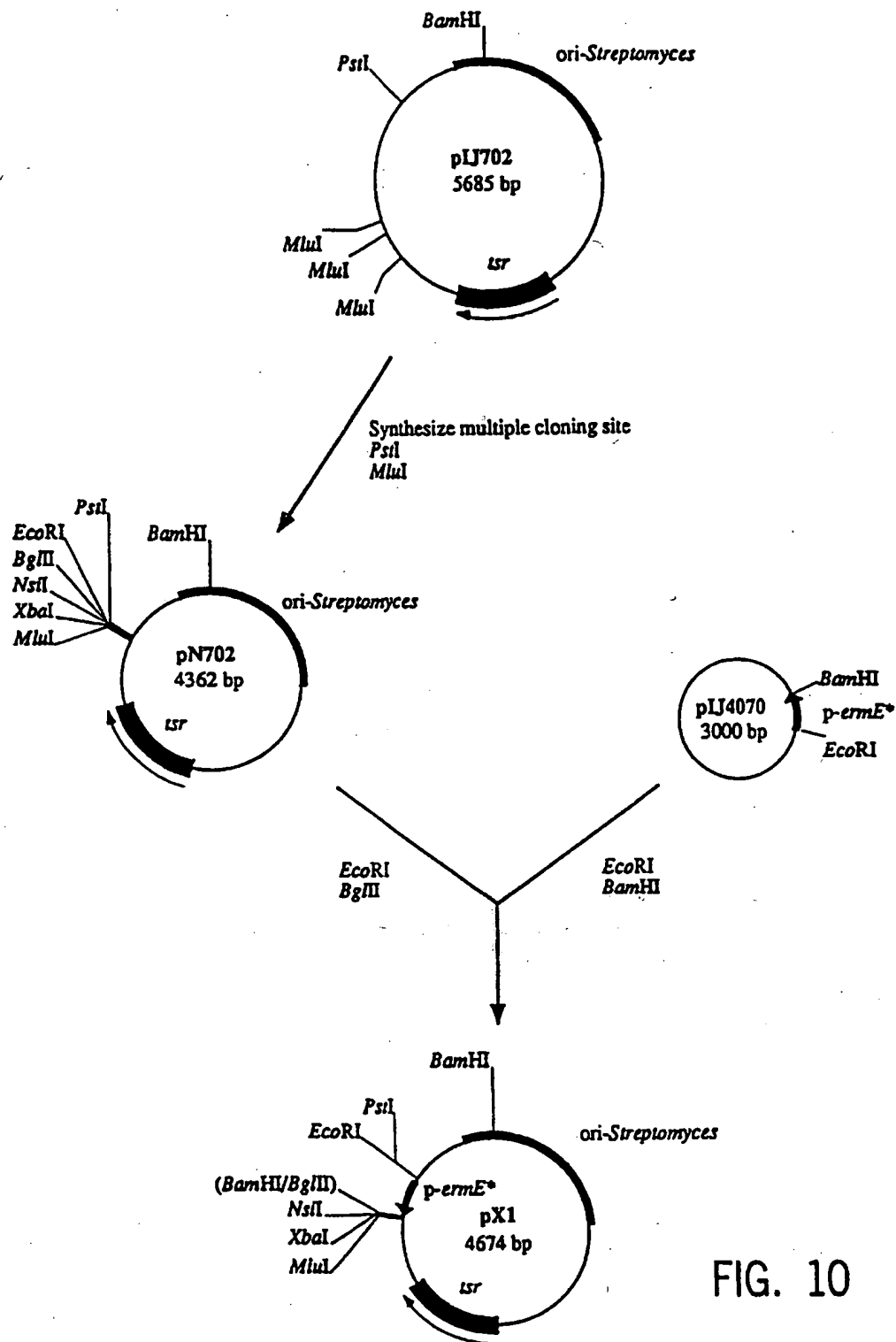


FIG. 10

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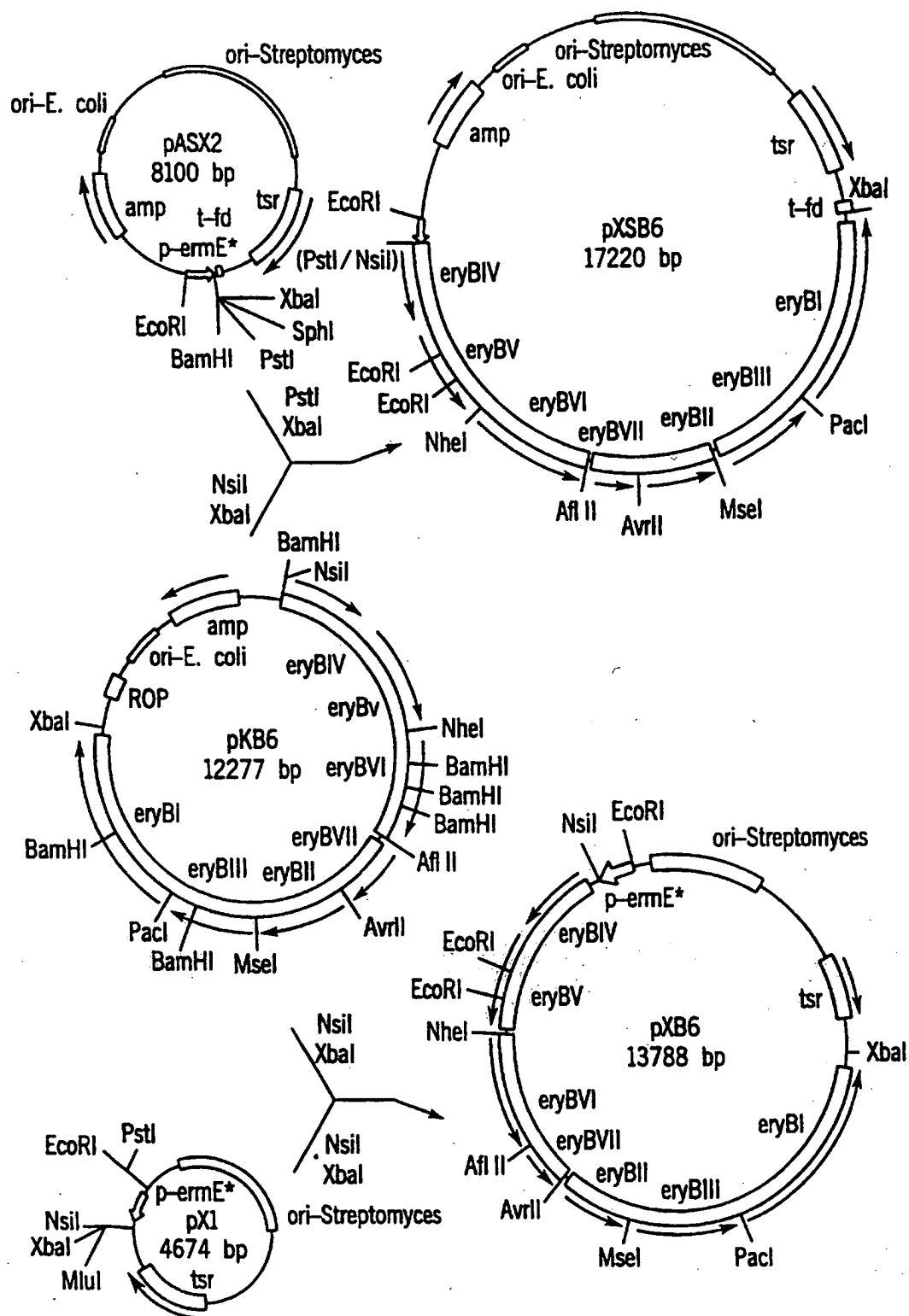


FIG. 11

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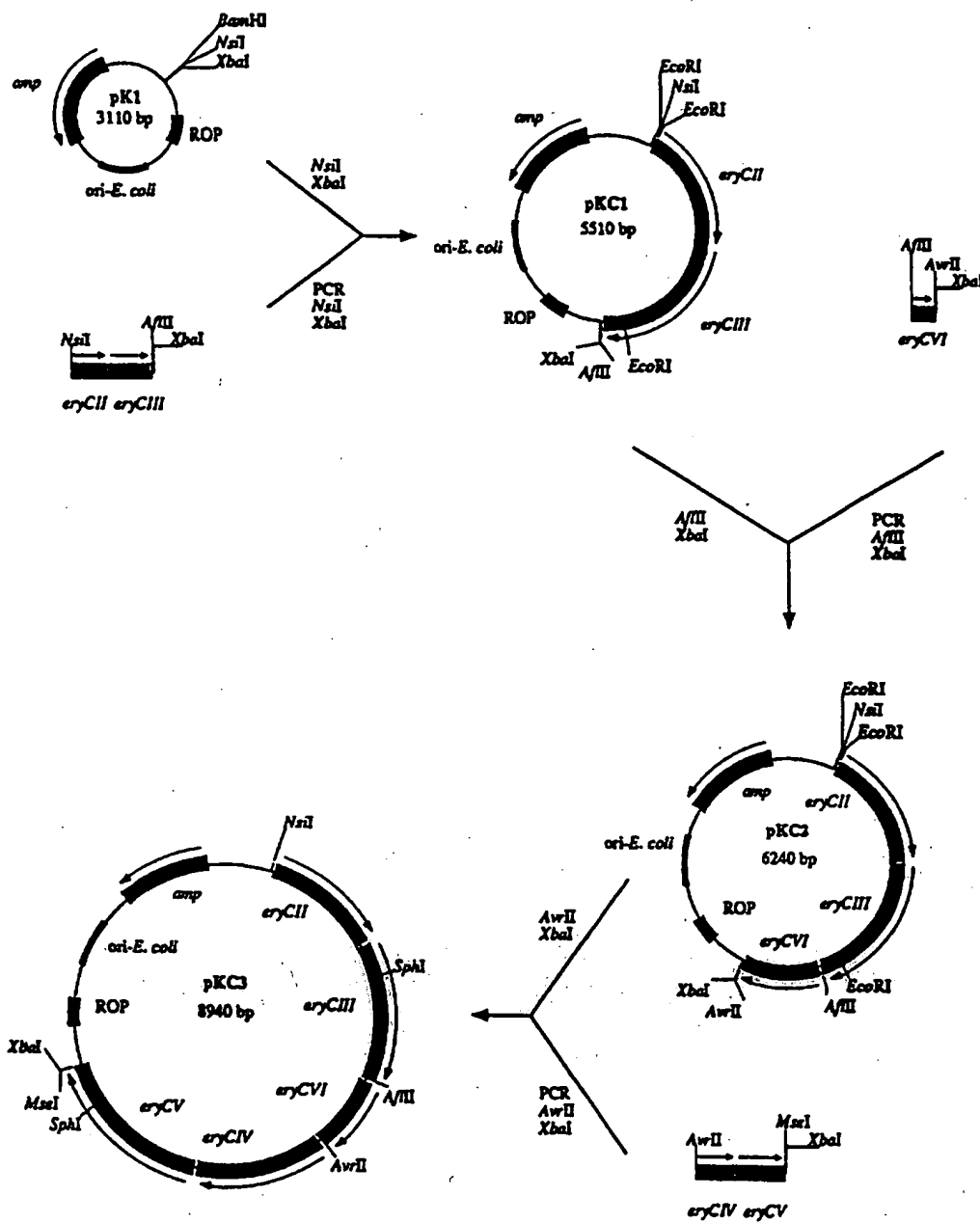


FIG. 12A

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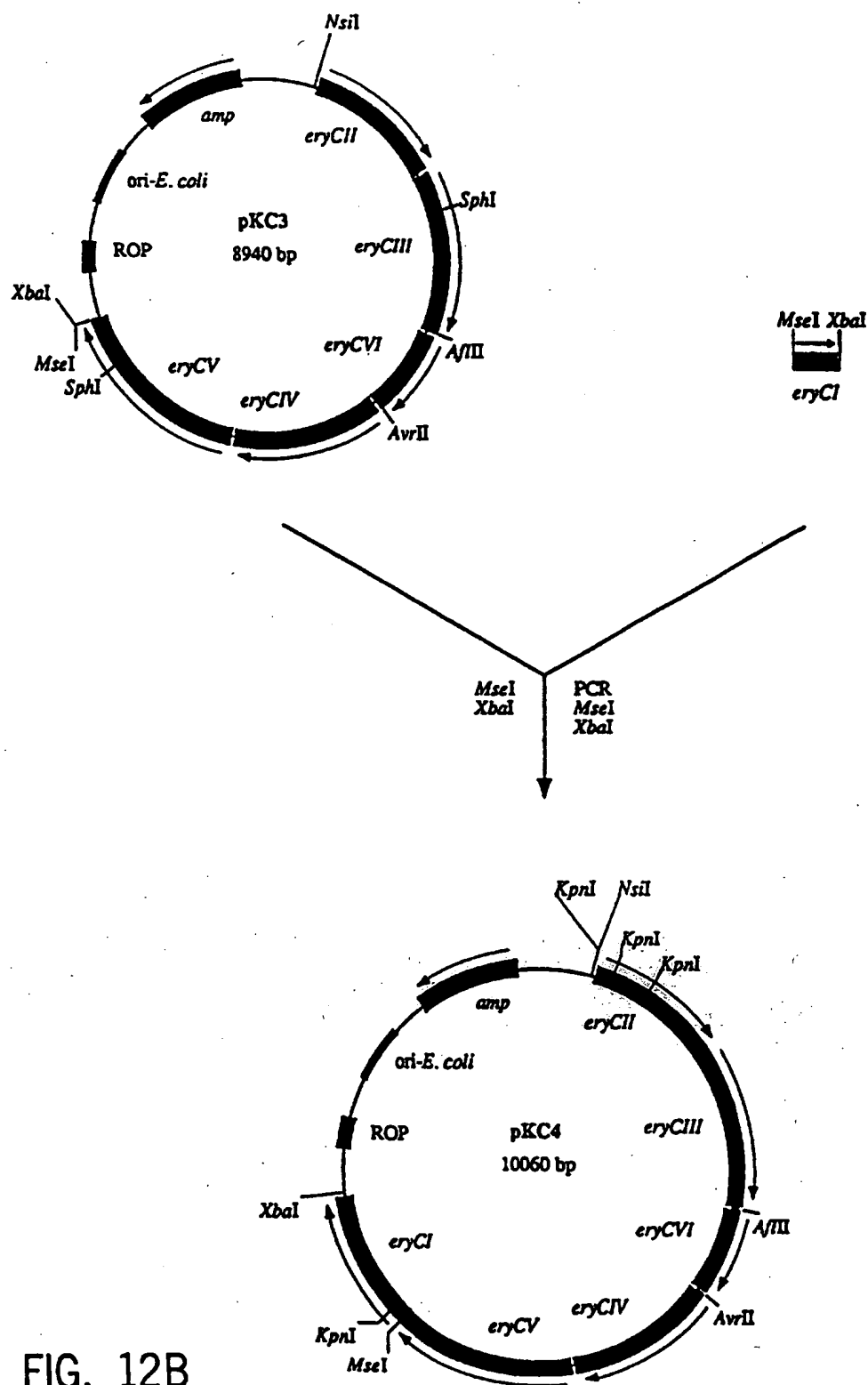


FIG. 12B

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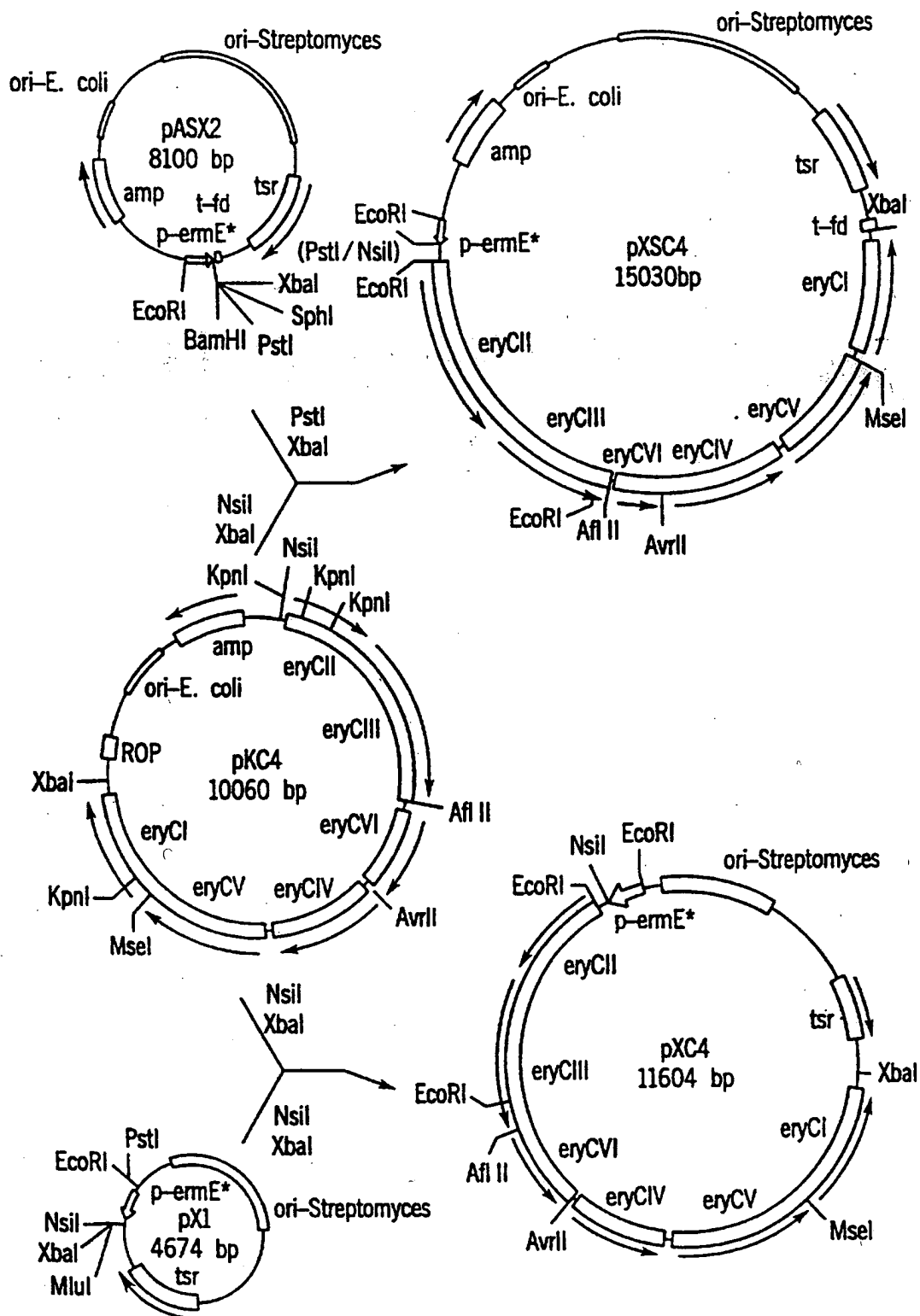


FIG. 13